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Molecular mechanisms underlying the development of tau pathology caused by tau fragmentation

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Molecular mechanisms underlying the development of tau pathology caused by tau fragmentation

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Thesis submitted in fulfilment of the degree of Doctor of Philosophy

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Declaration

I hereby declare that with the exception of the cell counting (Chapter 5 Figure 5.3D) all of the work presented in this thesis is my own.

Tong Guo

October 2016

Acknowledgements

It takes four years for an athlete to complete the Olympic journey. My “Olympic journey”, 2013 – 2016, has finally come to an end. Looking backward from the finishing line, I am clearly aware that it is not “I” who made it. In contrast, it is “we” who made it.

Firstly, I would like to thank my parents. Ten years ago, I started off as a freshman of Wuhan University, which is 850 miles away from my hometown. Since then, no matter how far I’ve gone, they have always stood behind me. We three are like a space shuttle: two rocket boosters burned every drop of fuel within them, so the shuttle can escape from gravity to explore the universe. I am very fortunate to be their son. They not only offered me the freedom from fear and want, but most importantly, the freedom to be myself. In China, a lot of children live a very confined and exam-oriented life. However, from the very beginning, my parents didn’t try to plan and manipulate every aspect of my life. On the contrary, they allowed me to explore, encouraged me to have my own thoughts, and respected my own decisions. When I set off on my own life adventure, they watched me quietly with caution and care like the catcher in the rye, but never interfered unless absolutely necessary.

My research journey in London began with an e-mail I sent to Diane Hanger, my first supervisor. Since then, she has put in a great effort to support me during the application process and throughout my whole PhD. When we talked on the phone for the first time, she asked about my previous lab experience. At that time, I was awkwardly trying to spell “SH-SY5Y cells” letter by letter, not knowing I could just say “sushi cells” for short. But now, I have finished my PhD thesis entirely in English, not to mention all the oral and poster presentations. On top of that, I succeeded in applying for my own research funding, my “first pot of gold in academia! Without her help and supervision, I wouldn’t have achieved any of this. What I learnt from her was not only language skills and knowledge, it was much more. She taught me how to think critically and strategically, to stay calm and see things from different perspectives. Most importantly, she gave her trust, patience, encouragement and tolerance to me. Under her leadership, I have been able to extend the boundaries of my research project, and develop myself as a professional researcher.

Thank you also to Wendy Noble, my second supervisor. Thank you for being so kind and approachable to me and especially for the wallet with a big “Edinburgh” on it. In “*My Wonderful Lousy Poem*”, Budd Schulberg wrote: “Those conflicting but

complementary voices of my childhood echo down through the years-wonderful, lousy, wonderful, lousy-like two powerful, opposing winds buffeting me. I try to navigate my little craft so as not to capsize before either. Between the two poles of affirmation and doubt, both in the name of love, I try to follow my true course.” It is so true for science, too. In the course of research “lousy” often dominates and I am glad to hear something like “well done” from you.

The very moment I saw Sebastien Paillusson, he was making coffee with his own coffee grinder. The aroma was so intense that I could smell it at the other end of the corridor. So far I am still so surprised and puzzled as to how this “Chinese-French” friendship worked out, as we are dramatically different. Unlike me, he is very outgoing and versatile, whereas I am quite introverted. Being a friend of his means I have to step out of my comfort zone, which is not always pleasant, but thanks to this, I was able to experience life in a different way. It is a path filled with things I don’t know and scenery I haven’t seen. Several times we travelled outside London, although every journey ended with two exhausted guys falling asleep on the night train, these memories are so unforgettable, just like taking a shot of scotch in the freezing winter.

Moreover, thank you to Amy Pooler, Beatriz Gomez Perez-Nievas, Gabor Morotz, Lizzie Glennon, Martina Hughes, Patricia Gómez Suaga, and Sarah Müller. Thank you for selflessly sharing your expertise with me. I would like to wish you all a successful career. Thank you to Dawn Lau, Teresa Rodriguez, and Emma Philips. You are the first people who welcomed me, and thank you for making our corner office so cosy and home-like. Thank you to Alan Stepto, Daniel Solomon, Cara Croft, Ksenia Kurbatskaya, Marie Bondulich, and Matthew Wade. I still miss the years we spent in the massive lab back in the IoPPN. A lot of fun would be lost without you. To Dina Dakkak, Natalia Yankova and Holly Barker, I wish you all the best for your PhD. Thank you to all my friends and colleagues in the Wohl, you are the best cure against loneliness!

Special thanks go to Dina Dakkak, Matthew Worssam, Yonji Lee and Kanchan Halai. Thank you for your effort during the completion of my research project and thesis. To Jenny Greig, Sarah Freckleton, and Naomi Hartopp, thanks for the advice on writing.

Finally, I would like to thank King's College London, Alzheimer's Research and UK King's College London Network Centre for funding my PhD as well as the work presented in this thesis. All the above would not be possible without your vision and generosity.

*There are places I remember
All my life though some have changed
Some forever not for better
Some have gone and some remain
All these places have their moments
With lovers and friends I still can recall
Some are dead and some are living
In my life I've loved them all*

*But of all these friends and lovers
There is no one compares with you
And these memories lose their meaning
When I think of love as something new
Though I know I'll never lose affection
For people and things that went before
I know I'll often stop and think about them
In my life I love you more*

-----Beatles "In My Life"

Tong Guo
October 2016

Abstract

The tauopathies are a group of neurodegenerative diseases characterised by pathological changes in tau protein along with synaptic dysfunction, breakdown in neuronal connectivity and neuronal cell death. Evidence has shown a link between tau fragmentation and the tauopathies. However, the role of this post translational modification in the development of disease is still unclear. This thesis describes the pathological consequences of N-terminal cleavage of tau in a cell model stably expressing a human brain-derived, 35 kDa tau fragment (Tau35). Compared to full length human tau protein, Tau35 displayed several loss-of-function defects such as reduced microtubule binding affinity, failure to regulate microtubule organisation, and reduced acetylation of tubulin. Inhibiting the phosphorylation of Tau35 does not enhance the microtubule binding affinity of Tau35, suggesting the role of the N-terminal half of tau in mediating the tau-microtubule interaction. Moreover, missing of the N-terminus of tau reduced but did not abolish the association between tau and cytoplasmic membrane. On the other hand, this tau fragment also gained a series of pathological features, including aberrant phosphorylation, but insoluble tau aggregates are absent. Meanwhile, expression of Tau35 triggered activation of the unfolded protein response in cells, and disrupted the regulation of the Akt-GSK3 β pathway. My results therefore revealed that apart from the long-proven importance of microtubule binding repeats in the C-terminal half of tau in mediating tau-microtubule interactions, the N-terminus of tau is also involved in these processes. Moreover, after deletion of the N-terminus, the resultant tau fragment, Tau35 not only loses the ability to execute the physiological function of tau as a microtubule stabiliser, but also interrupts the unfolded protein response and GSK3 β pathways which in turn drive further pathological changes of Tau35. In summary, my findings provide a greater understanding of the role of tau fragmentation in the progression of tau pathology, and the novel Tau35 cell model will aid the understanding of disease-associated mechanisms and the development of novel treatments for human tauopathies.

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List of Abbreviations

AD	Alzheimer's disease
AEP	Asparagine endopeptidase
Akt/PKB	Protein kinase B
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
ApoE3	Apolipoprotein E3
APP	Amyloid precursor protein
APS	Ammonium persulfate
ASK1	Apoptosis signal-regulating kinase 1
ATA-1	Alpha Tubulin Acetyltransferase
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
A β	Amyloid-beta peptide
Bcl-2	B-cell lymphoma 2
C9ALS	ALS with the open reading frame 72 on chromosome 9 repeat expansion
CA	Corpora amylacea
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
CBD	Corticobasal degeneration
Cdk5	Cyclin-dependent kinase
CHIP-HSP90	C-terminus of heat shock protein 70-interacting protein-heat shock protein 90
CHO	Chinese hamster ovary
CHOP	CCAAT-enhancer-binding proteins homologous protein
CK1	Casein kinase I
CNS	Central nervous system
CREB	cAMP-response element binding protein
CSF	Cerebrospinal fluid
CT	Computed tomography
CTE	Chronic traumatic encephalopathy
DCTN1	Dynactin subunit 1
DCTN2	Dynactin subunit 2
DMSO	Dimethyl sulfoxide
DTT	1,4-Dithiothreitol

DYRK1A	Dual specificity tyrosine-phosphorylation-regulated kinase 1A
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	ethylene glycol-bis (β -aminoethyl ether)-N,N,N',N'-tetraacetic acid
eIF2 α	Eukaryotic initiation factor 2 α
ER	Endoplasmic reticulum
ERAD	Endoplasmic-reticulum-associated protein degradation
FBS	Foetal bovine serum
FDG-PET	^{18}F -fluorodeoxyglucose positron emission tomography
fMRI	Functional magnetic resonance imaging
FoxO1	Forkhead box protein O1
FTDP-17	Frontotemporal dementia and parkinsonism linked to chromosome 17
FTLD-tau	frontotemporal lobar degeneration-tau
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCI	Gyrus cinguli
GFP	Green fluorescent protein
Grb2	Growth factor receptor-bound protein 2
GRP	Glucose-regulated protein
GSK3 α/β	Glycogen synthase kinase 3 α/β
GVD	Granulovacuolar degeneration
HBSS	Hank's balanced salt solution
HD	Huntington's disease
HDAC6	Histone deacetylase 6
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hHRD1	Human 3-hydroxy-3-methylglutaryl-coenzyme A reductase degradation 1
ICC	Immunocytochemistry
IKK	I κ B kinase
IL-1 α	Interleukin 1 α
IL-1 β	Interleukin 1 β
IR	Insulin receptor
IRE1	inositol-requiring transmembrane kinase/endonuclease
IRS	Insulin receptor substrates

ISR	Integrated stress response
JIP1	JNK-interacting protein 1
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
Ki	Inhibitory constant
LB	Luria Bertani broth
LC-3II	Microtubule-associated protein 1 light chain 3 alpha II
LRRK2	Leucine-rich repeat kinase 2
LTD	Long-term depression
LTP	Long-term potentiation
MAP	Microtubule-associated protein
MAPK	Mitogen-activated protein kinase
MAPT	Microtubule-associated protein tau
MARKs	Microtubule affinity-regulating kinase
MEC-17	Mechanosensory abnormality-17
MRI	Magnetic resonance imaging
MT	Microtubule
MTBD	Microtubule binding domain
mTOR	Mammalian target of rapamycin
N2a	Neuro2a
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NT2	NTera-2
NTF	Neurofibrillary tangle
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's disease
PDI	Protein disulfide isomerase
PERK	Protein kinase R-like endoplasmic reticulum kinase
PET	Positron emission tomography
PFA	Paraformaldehyde
PHF	Paired helical filament
PI3K	Phosphoinositide 3-kinase
PiB	Pittsburgh compound B
PiD	Pick's disease
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	cAMP-dependent protein kinase

PKC	Protein kinase c
PKN	Protein kinase N
PLC γ	Phosphoinositide phospholipase γ
PP	Protein phosphatase
PSA	Puromycin-sensitive aminopeptidase
PSD-95	Postsynaptic density protein-95
PSEN-1	Presenilin-1
PSP	Progressive supranuclear palsy
RNase	Ribonuclease
rpm	revolutions per minute
S.E.M	Standard error of the mean
s/v CJD	Sporadic/variant Creutzfeldt–Jakob disease
S1P	Site 1 protease
S2P	Site 2 protease
SB	Laemmli sample buffer
SCAMP5	Secretory carrier membrane protein 5
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH-3	The SRC Homology 3 Domain
SIRT1	Sirtuin 1
SOD1	Superoxide dismutase 1
SUMO-1	Small ubiquitin-like modifier protein-1
sXBP1	spliced X-box binding protein 1
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline-Tween 20
TDP-43	Transactive response DNA-binding protein 43
TEMED	Tetramethylethylenediamine
TNF- α	Tumor necrosis factor α
TRAF	Tumour necrosis factor receptor-associated factor
TRAF2	Tumor necrosis factor receptor-associated factor 2
TRB3	tribbles-related protein 3
TTBK	Tau tubulin kinases
Tween 20	Polyethylene glycol sorbitan monolaurate 20
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system
VMA21	Vacuolar ATPase assembly integral membrane protein
XBP1	X-box binding protein 1

Symbols for amino acid

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Cys	Cysteine
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

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Publications arising from this thesis

Bondulich MK, Guo T, Meehan C, Manion J, Rodriguez Martin T, Mitchell JC, Hortobagyi T, Yankova N, Stygelbout V, Brion JP, Noble W, Hanger DP. (2016). Tauopathy induced by low level expression of a human brain-derived tau fragment in mice is rescued by phenylbutyrate. *Brain*.139(Pt 8):2290-306.

Hanger DP, Lau DH, Phillips EC, Bondulich MK, Guo T, Woodward BW, Pooler AM, Noble W. (2014). Intracellular and extracellular roles for tau in neurodegenerative disease. *Journal of Alzheimer's Disease*. 2014;40 S37-45

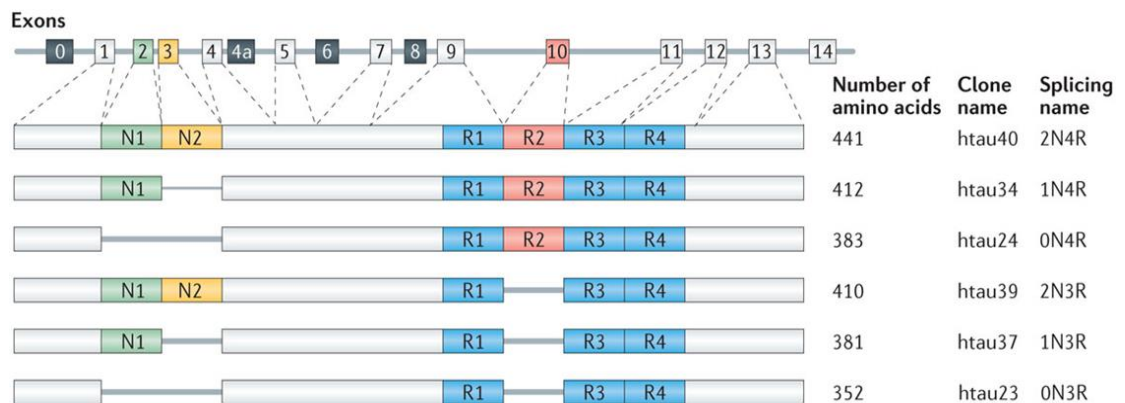
Chapter 1 Introduction

1.1 Tau: from gene to protein

Tau protein was first identified in the mid-1970s and named “tubulin-associated unit” (tau) because of its ability to bind to and stabilise microtubules (Cleveland et al., 1977; Weingarten et al., 1975; Witman et al., 1976).

Human tau is encoded by the Microtubule-associated protein tau (*MAPT*) gene, which comprises 16 exons and is located on chromosome 17q21 (Andreadis, 2006). The *MAPT* gene is subjected to alternative splicing of exons 2, 3 and 10 (E2, E3 and E10, respectively). The presence and absence of E2 and E3 determines the number of 29-residue amino-terminal inserts present in these isoforms: isoforms containing 0, 1 or 2 inserts are known as 0N, 1N and 2N, respectively. In parallel, E10 encodes the second of four microtubule binding repeat domain (MTBD) and determines whether tau isoforms contain three or four MTB repeat domains which are identified as the structural basis for microtubule binding (3R or 4R, respectively) (Lee et al., 1988). Hence, the six isoforms of tau in the human central nervous system (CNS) can be catalogued as a combination of 0N, 1N or 2N and 3R or 4R, each containing between 352 and 441 amino acids (Fig 1.1). The shortest tau isoform 0N3R, has an apparent molecular weight on SDS-PAGE of 48 kDa, and the longest isoform, 2N4R, is 67 kDa (Crowther et al., 1989; Lee et al., 2001). The expression of tau is developmentally regulated and varies between different species: in the adult human brain, six isoforms of tau are expressed, whereas in foetal brain, only the shortest tau isoform is expressed. The 3R and 4R forms are approximately equally expressed in normal adult human brain and the 2N isoform is underrepresented in comparison with the 0N and 1N isoforms. The 0N, 1N and 2N tau isoforms comprise ~37%, ~54% and ~9% of total tau, respectively (Goedert and Jakes, 1990). However, adult mouse brain expresses almost exclusively the three isoforms of 4R tau (Kosik et al., 1989b; Takuma et al., 2003), and 3R tau is only transiently expressed in the neurons of foetal and newborn mice (Llorens-Martin et al., 2012). Mouse and human tau proteins are highly homologous with 92% sequence similarity, but they differ considerably over the N-terminus, where there is only 57% sequence similarity (Adams et al., 2009; Goedert et al., 1988). Splicing of *MAPT* also exhibits regional differences in the brain and for example, within human brains, the amount of 0N3R tau is lower in the cerebellum than it is in other brain regions (Boutajangout et al., 2004; Trabzuni et al., 2012).

Figure 1.1 Gene and structure of tau

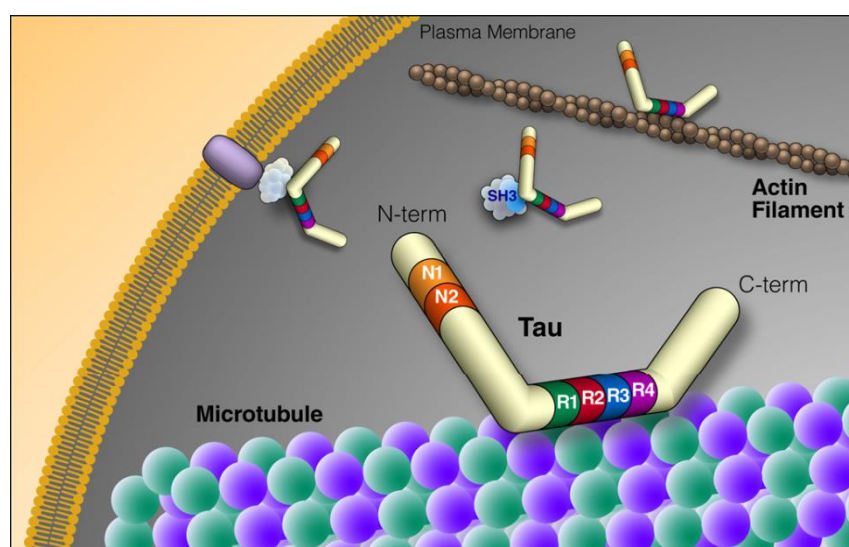


MAPT, the gene encoding human tau, contains 16 exons (Neve et al., 1986). Exons 1, 4, 5, 7, 9, 11, 12 and 13 are constitutively expressed, whereas the other exons are subject to alternative splicing. The six human brain tau isoforms are generated through alternative splicing of exons 2, 3 and 10 (Andreadis, 2006). These tau isoforms differ according to the presence of 0, 1 or 2 amino-terminal inserts (0N, 1N or 2N, respectively) and the presence of microtubule-binding repeat R2, encoded by E10, yielding 3 or 4 carboxy-terminal repeat domain (3R or 4R, respectively) tau species (Wang and Mandelkow, 2016).

Tau is a natively unfolded protein (Mukrasch et al., 2009). The 2N4R tau isoform contains 80 Ser or Thr residues, 56 negatively charged (Asp or Glu) residues, 58 positively charged (Lys or Arg) residues and 8 aromatic (5 Tyr and 3 Phe, but no Trp) residues. Tau is unusually stable under acidic conditions and in high temperatures. Overall, tau is a basic protein; however, the ~120 N-terminal residues are predominantly acidic, and the ~40-residue C-terminus is approximately neutral (Wang and Mandelkow, 2016). Tau protein comprises four major domains: (1) an acidic projection region towards the N-terminus; (2) a proline-rich region in the centre of the molecule; (3) a microtubule-binding region, which contains either three or four carboxy-terminal repeat domains mediating the binding between microtubule and (4) a C-terminal tail (Mandelkow et al., 1996). All four regions are required to execute the physiological function of tau. Tau binds to microtubules, with the N- and C-terminal regions of tau projecting outwards due to their charge (Santarella et al., 2004). The N-terminal region of tau can interact with cell membranes, thus regulating the spacing between microtubules (Frappier et al., 1994; Maas et al., 2000) and possibly also mediating the physiological secretion of tau into the

extracellular space (Pooler and Hanger, 2010; Pooler et al., 2013; Pooler et al., 2012). Moreover, the proline-rich domain of tau harbours many phosphorylation sites and this region can bind to Src homology-3 (SH3) domains of other proteins including the non-receptor-associated tyrosine kinase Fyn (Lau et al., 2016; Reynolds et al., 2008). This indicates a role for tau in regulating the intracellular localisation of Fyn, and potentially signals transduction in neurons.

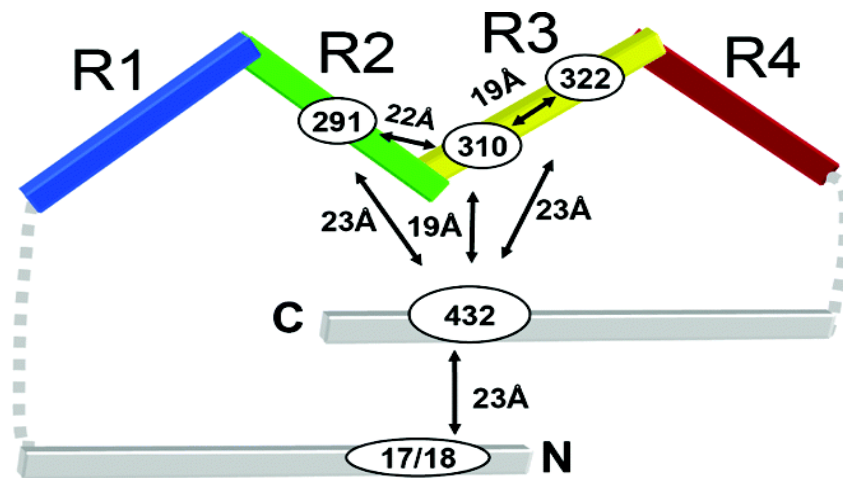
Figure 1.2 Tau structure and function



Tau is an intrinsically disordered protein. The domains of tau have different binding partners, thereby executing different functions. The diversity of tau functions and binding partners is highlighted in Section 1.4. N-term: N-terminus; C-term, C-terminus; SH3, protein Src-homology 3 domain (Morris et al., 2011).

Differences in the biochemical and structural properties between tau isoforms affect the function of tau directly. Owing to the additional microtubule-binding repeat domain encoded by E10, 4R tau displays a higher affinity for microtubules than does 3R tau, and is therefore more efficient at promoting microtubule assembly (Goedert and Jakes, 1990). In recent years, intensive research has provided more molecular details of the function of the N1 and N2 inserts. From a structural biology standpoint, despite its unfolded character, the tau molecule in solution shows a preference for a paperclip-like conformation (Fig 1.3) , in which the N-terminal, C-terminal and the repeat domains all approach each other (Jeganathan et al., 2006).

Figure 1.3 Model of the intramolecular interactions within soluble tau



The polypeptide chain of four-repeat tau is shown with different coloured repeats (R1, blue; R2, green; R3, yellow; R4, red) and the adjacent N- and C-terminal sequences in grey. Residue 17 or 18, near the N-terminus and residue 432 or 435, near the C-terminus of tau are close to each other. Residues in the repeat domain (residue 291 in R2 and residues 310 and 322 in R3) are close to each other and the C-terminus of tau (Jeganathan et al., 2006).

As part of projection domain of tau, the two N-terminal inserts can influence the attachment or spacing between microtubules and other cell components (Goedert and Jakes, 1990; Violet et al., 2014). In addition, these two N-terminal inserts might affect the subcellular distribution of tau in neurons as, in mice, the 0N, 1N or 2N tau isoforms each show distinct subcellular distributions (Liu and Götz, 2013). Moreover, distinct binding partners of tau display preferences towards different tau isoforms, as revealed by co-immunoprecipitation with tau isoform-specific antibodies. For example, Apolipoprotein A1 (Apo A1) displayed a five-fold preference for interaction with 2N tau isoforms, whereas β -synuclein showed a preference for 0N tau isoforms (Liu et al., 2016).

Tau is mainly found in neurons but is also present at low levels in glia, and has also been detected outside cells (Goedert et al., 1996; Goedert et al., 1989; Lopresti et al., 1995; Wang and Mandelkow, 2016). The subcellular distribution of tau changes according to the stage of neuron development (Drubin et al., 1984). In nascent neurons, tau distributes evenly in the cell body and neurites. Later, when axons

emerge and neurons become polarised, tau is enriched in axons and exhibits a proximal-distal increase with minor amounts found in dendrites and also in nuclei (Black et al., 1996; Kempf et al., 1996; Mandell and Banker, 1996; Papasozomenos and Binder, 1987; Sultan et al., 2011).

Although the processes underlying the polarised distribution of tau in adult neurons are not fully elucidated, possible mechanisms at both the mRNA and protein level have been suggested. Tau mRNA tends to be distributed towards proximal axons, and this is mediated by a 3' untranslated region axonal localisation signal (Aronov et al., 2001; Litman et al., 1993). Furthermore, tau mRNA is preferentially translated in axons (Morita and Sobuě, 2009). At the tau protein level, several factors may contribute to its axonal localisation, including a faster turnover of tau in the somatodendritic compartment than in axons; the higher affinity of tau for microtubules in axons, compared to dendrites (Hirokawa et al., 1996); fast axonal transport of tau soon after its synthesis in the cell body (Kosik et al., 1989a); and an axon initial segment barrier against the retrograde diffusion of tau into dendrites (Li et al., 2011). Notably, the sorting of tau seems to be isoform-dependent, as different tau isoforms show distinctive distributions in different compartments (Liu and Götz, 2013).

1.2 Tau mediated neurodegenerative disease: tauopathies

Intensive study into sporadic and familial neurodegenerative diseases over the past decade has led to the realisation that, although many of these disorders are characterised by distinct hallmark brain lesions, one similarity is the formation of filamentous deposits of abnormal proteins (Takalo et al., 2013). Thus, a group of heterogeneous dementias and movement disorders, neuropathologically characterised by prominent intracellular accumulations of abnormal filaments that form neurofibrillary tangles (NTFs), formed of tau, as well as other tau inclusions in neurons and glia, are collectively defined as neurodegenerative tauopathies (Table 1.1). The importance of abnormal tau in disease onset and/or progression is unequivocally proven by the discovery of multiple tau gene mutations in neuropathology of frontotemporal lobar degeneration-tau (FTLD-tau), showing that tau abnormalities alone are sufficient to cause neurodegenerative disease (Foster et al., 1997; Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). This seminal finding paved the way for further investigation of the role of tau in brain dysfunction and neurodegeneration.

Table 1.1 Tau-mediated neurodegenerative diseases

Neurological disease	Description	Tau Abnormality	References
FTLD-tau	Frontotemporal atrophy is the primary feature, with neuronal loss, gliosis, and cortical spongiform changes in the lobes.	This disorder is the consequence of mutations in the tau gene with an extensive variety of clinical symptoms.	(Avila et al., 2004; Ludolph et al., 2009)
Progressive supranuclear palsy	Clinical manifestation includes prominent postural instability supranuclear vertical gaze palsy, pseudobulbar palsy, levodopa-unresponsive parkinsonism, and frontal cognitive disturbances	The neuropathological characteristics include midbrain atrophy as a consequence of tau inclusions (such as NFT) in neurons, astrocytes, and oligodendrocytes; a few cases of familial origin have mutations in the tau gene.	(Dickson et al., 2007; Tawana and Ramsden, 2001; Williams and Lees, 2009)
Corticobasal degeneration	Clinical manifestation includes dementia, movement disorder, cortical sensory loss, oculomotor dysfunction, and dysphagia.	The principal lesions of this disease are cortical and nigral atrophy as a consequence of tau inclusions (only 4R isoforms) in astrocytic plaques as an exclusive feature of this disorder.	(Ludolph et al., 2009)
Guam parkinsonism dementia complex	This form of dementia is characterised by a progressive decline of intellectual faculties, disorientation and behavioural changes. Also rigidity and postural deformities, hyperreflexia, and spinal muscular atrophy.	This disorder is characterised by cortical atrophy, neuronal loss and extensive NFTs in the neocortex and hippocampus.	(Murakami, 1999; Winton et al., 2006)

Niemann–Pick type C	A dementia with a sphingolipid storage disorder as a result of inherited deficiencies of intracellular lipid trafficking proteins, which lead to accumulation of cholesterol and glycosphingolipids in late endosomes and lysosomes.	The neuropathological features include numerous au-positive NFTs and neuropil threads in the midbrain and cerebral cortex.	(Ludolph et al., 2009; Pacheco and Lieberman, 2008)
Dementia pugilistica	A variant of chronic traumatic encephalopathy (CTE), a serious type of brain damage resulting from repeated concussions. This disorder involves pathological processes which affect parts of the brainstem, the cerebellum and the cerebral hemispheres.	Degeneration of the substantia nigra, cerebellar scarring, and partial disintegration of the septum pellucidum and the widespread presence of NFT without neuritic plaques, particularly in the medial temporal cortex.	(Adams and Bruton, 1989)
Pick's disease	Frontotemporal dementia that results in disturbances in language and behaviour and is associated with frontal lobe atrophy.	This disease is characterised by the presence of cytoplasmic highly phosphorylated tau (only 3R isoforms) inclusions in neurons of the frontal lobe, known as Pick's bodies.	(Avila et al., 2004; Robert and Mathuranath, 2007)

The table describes a number of key neurodegenerative disorders in which tau inclusions have been identified in the brain; NFT: neurofibrillary tangles; AD: Alzheimer's disease; PHFs: paired helical filaments; CTE: Chronic traumatic encephalopathy; (Meraz-Ríos et al., 2010).

1.2.1 Tauopathies linked to tau mutations

To date, several pathogenic events have been shown to be caused, either directly or indirectly, by abnormal changes in tau. Amongst these, mutations in the tau gene (*MAPT*) have been documented in genetic studies as a direct cause of FTLD–tau (Goedert and Jakes, 2005; von Bergen et al., 2001). All cases of FTLD-tau are characterised by the presence of filamentous inclusions that are composed of highly phosphorylated tau. The protein products of many tau mutations display altered

biochemical properties, including (1) a predisposition to assemble into filaments and are therefore able to undergo rapid fibrillisation (Goedert and Jakes, 2005; Nacharaju et al., 1999); (2) a tendency to be more readily phosphorylated and/or less prone to dephosphorylation (Alonso et al., 2004); or (3) show impaired microtubule binding properties (Dayanandan et al., 1999; Hong et al., 1998). Contrastingly, intronic *MAPT* mutations, and most coding-region mutations in E10 of tau (e.g., N279K, L284L, N296, N296N, N296H, S305N and S305S), may instead perturb the alternative splicing of tau, disrupting the normal one-to-one ratio of the 3R to 4R tau isoforms (Goedert and Jakes, 2005).

1.2.2 Amyloid- β induced tau pathology

In Alzheimer's disease (AD), connections between amyloid- β (A β)-mediated toxicity and tau pathology have repeatedly been proposed. However, understanding of the mechanisms that link A β and tau deposition is not complete. One hypothesis for the pathogenesis of AD proposes that the development of neurodegenerative conditions in AD depends on A β working in concert with tau, which is described in further detail below.

Structural biology studies have revealed that the dominant components of NFTs in AD are paired helical filaments (PHFs) and straight filaments (Kidd, 1963). Both PHFs and straight filaments are composed predominantly of abnormally phosphorylated tau protein. This was initially reported in 1985 (Brion et al., 1985), and are confirmed by subsequent studies (Goedert et al., 1988; Kondo et al., 1988; Kosik et al., 1988; Lee et al., 1991; Wischik et al., 1988a). PHFs purified from AD brain can be resolved into three major bands of approximately 68, 64, and 60 kDa, as well as a minor band of approximately 72 kDa using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (Greenberg and Davies, 1990; Lee et al., 1991). A similar band pattern to that produced by six isoforms of tau found in adult human brain was exhibited after the dephosphorylation of PHF samples (Goedert et al., 1992; Greenberg et al., 1992; Lee et al., 1991). PHFs have been shown to consist of both 3R and 4R tau isoforms in a one-to-one ratio, similar to the isoform composition of tau in normal adult human brain (Goedert et al., 1995; Hong et al., 1998; Morishima-Kawashima et al., 1995; Trojanowski and Lee, 1994).

1.2.3 Tauopathies independent of amyloid- β

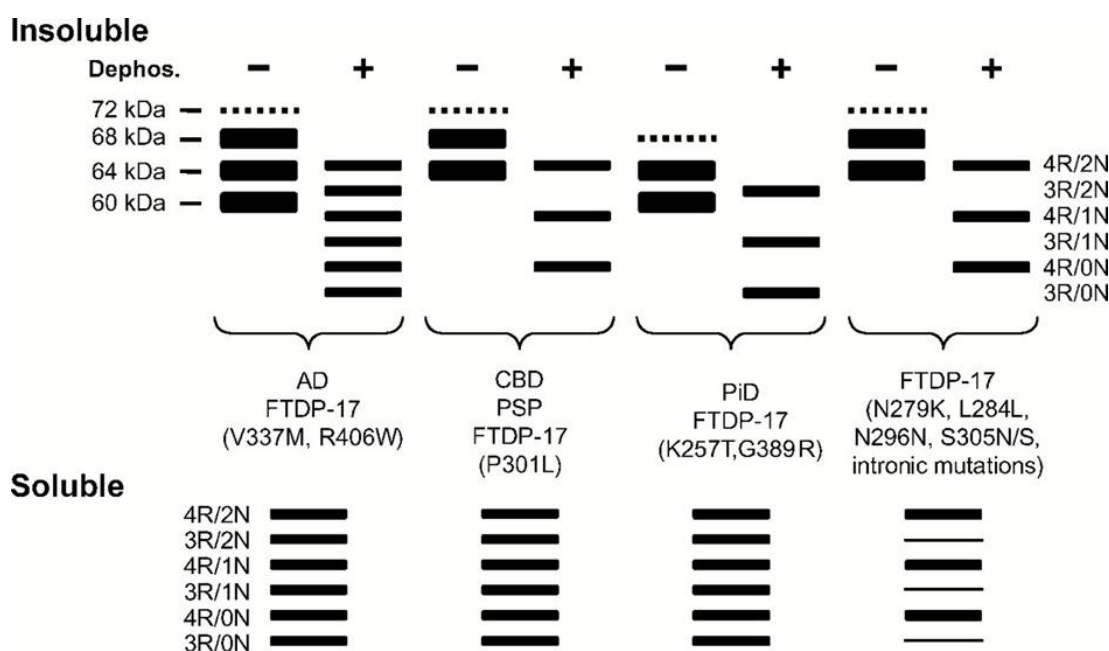
Importantly, studies of tauopathies other than AD have shown abundant filamentous tau pathology and brain degeneration in the absence of extracellular amyloid deposits. Such disorders include progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and Pick's disease (PiD) (Table 1.1).

PSP is clinically characterised by supranuclear gaze palsy as well as by prominent postural instability (Steele et al., 1964). From a neuropathological perspective, PSP is characterised by atrophy of the basal ganglia, subthalamus, and brainstem, with corresponding neuronal loss and gliosis. A high density of fibrillary tau pathology, including neuropil threads and round or globose neurofibrillary tangles, is present in affected brain regions (Hauw et al., 1994; Litvan et al., 1996; Pollock et al., 1986). Glial fibrillary tangles are frequently present in both astrocytes (tufted astrocytes) and oligodendrocytes (coiled bodies) (Hauw et al., 1990; Komori, 1999; Yamada et al., 1992). Furthermore, insoluble, highly phosphorylated tau has been identified in the filamentous tau pathology of PSP. Despite the diverse fibril morphologies, the same profile of phosphorylation-dependent tau epitopes as tau protein detected in PHF from AD brains has been found (Schmidt et al., 1996). However, in contrast to the three major bands and one minor band identified in AD, only the two higher molecular weight major bands (68 and 64 kDa) and the minor 72 kDa band are present in PSP brain samples (Flament et al., 1991; Vermersh et al., 1994). These bands are made of hyperphosphorylated 4R tau isoforms (Sergeant et al., 1999; Spillantini et al., 1997). Furthermore, in PSP, the relative abundance of tau mRNA encoding 4R isoforms is increased in the brainstem but not in the cortex, in line with the distribution of the neurofibrillary pathology (Chambers et al., 1999).

CBD is an adult-onset progressive neurodegenerative disorder targeting the cerebral cortex, deep cerebellar nuclei, and substantia nigra, accompanied by prominent neuronal achromasia (Rebeiz et al., 1968; Rebeiz et al., 1967). The neuropathological hallmark of CBD includes depigmentation of the substantia nigra, as well as asymmetric frontoparietal atrophy. Extensive accumulation of tau-immunoreactive neuropil threads throughout grey and white matter is recognized as another feature of CBD (Feany et al., 1995; Feany et al., 1996). Neuronal loss with spongiosis, gliosis, and prominent glial and neuronal filamentous tau pathology is also observed (Iwatsubo et al., 1994; Mori et al., 1994). The glial tau pathology displays characteristic astrocytic plaques (Feany and Dickson, 1995), as well as numerous tau-immunoreactive inclusions in the white matter in both astrocytes and

oligodendrocytes (coiled bodies) (Komori, 1999; Komori et al., 1998). The tau filaments in CBD consist of both PHF-like and straight tubules (Komori, 1999; Ksiezak-Reding et al., 1994). The biochemical profile of insoluble tau in CBD resembles that of PSP in that it consists of two major bands of 64 and 68 kDa and a variable, minor band of 72 kDa. Sergeant and colleagues demonstrated that the fibrillary inclusions in CBD are composed predominantly of 4R tau isoforms (Sergeant et al., 1999). These findings, combined with the genetic similarity between PSP and CBD described by Di Maria and colleagues, strongly suggest that there is a substantial overlap between PSP and CBD pathology (Di Maria et al., 2000). This is also apparent with respect to the clinical (Hauw et al., 1994) and pathological (Feany et al., 1996) features. However, the possibilities that PSP and CBD are distinct nosological entities occurring in patients with similar genetic predisposition cannot be ruled out (Dickson, 1999; Scaravilli et al., 2005).

Figure 1.4 Schematic representation of the banding patterns of tau from different tauopathies analysed by western blot



Before dephosphorylation, insoluble tau from Alzheimer's disease (AD) brain and some frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) with mutations outside exon 10 (V337M and R406W), are resolved into three major bands and a minor, variable band. When dephosphorylated, tau resolves into six bands that correspond to recombinant tau. The soluble fraction contains all six tau isoforms. Major tau bands are depicted by solid bars and the thickness of the bars correlates with the relative abundance of

the specific tau isoform. A dashed bar is used to depict the minor, more variable, tau species (Lee et al., 2001).

PiD is a frontotemporal lobar and limbic atrophy associated with marked neuronal loss, spongiosis, and gliosis, with ballooned neurons and Pick's bodies (Brun et al., 1994; Dickson, 1998). Pick's bodies are reactive to antibodies against phosphorylated tau and are distributed mostly in layers II and VI of the neocortex and in the dentate granule neurons of the hippocampus (Iwatsubo et al., 1994; Probst et al., 1996). From a structural biology standpoint, Pick's bodies are composed of a mixture of wide, straight filaments and wide, long-period twisted filaments (Dickson, 1998; Munoz-Garcia and Ludwin, 1984; Murayama et al., 1990). Western blot analyses have revealed that the insoluble tau in PiD is distinct from that in AD, CBD, and PSP in that it comprises two major bands of 60 and 64 kDa and a variable, minor band of 68 kDa (Buée-Scherrer et al., 1996; Delacourte et al., 1996; Lieberman et al., 1998). It is believed that the two major PiD tau bands correspond to 3R tau isoforms, hence, PiD is regarded as a disease caused by overexpression of 3R tau (Mailliot et al., 1998; Sergeant et al., 1997).

In summary, although investigations into tau pathology mediated by tau mutations and A β have greatly enriched the knowledge of the tauopathies, discoveries of tauopathies independent of A β and tau mutations have necessitated a more comprehensive understanding of the properties of tau. Moreover, studies into PSP, CBD and Pick's disease show that the conversion of physiological tau to pathological tau plays a central role in the development of neurodegenerative disease.

1.3 The physiological role of tau

Tau was originally identified as a microtubule-associated protein. However, decades of intensive research have extended the knowledge of tau protein from its primary role as a microtubule stabiliser into a multi-functional protein which has various interaction partners. Tau also binds to and bundles actin filaments, thus modifying the organisation of the actin cytoskeleton (Fulga et al., 2007; He et al., 2009; Kotani et al., 1985). Tau binding to actin is mediated primarily by its microtubule-binding domain (Farias et al., 2002; Yu and Rasenick, 2006) and assisted by the adjacent proline-rich domain (He et al., 2009). The phosphorylation of tau appears to alter its association with actin, as tau phosphorylated at the KXGS motifs tends to colocalise

with actin filaments in growth cones during development and in rod-like inclusions of cofilin and actin (Whiteman et al., 2009). Since it can bind to both tubulin and actin, it is possible that tau connects the microtubule and actin filament networks (Farias et al., 2002)(Table 1.2). Notably, as described above, tau exhibits different functions in different subcellular compartments (Morris et al., 2011).

1.3.1 Tau in cytoskeleton dynamics

In adult neurons, tau mainly distributes into axons, where it interacts with microtubules via residues within the repeat-domain, while residues that lie between the microtubule-binding sites remain flexible (Kadavath et al., 2015). Upon binding, tau stabilises microtubules, promotes microtubule assembly and, in particular, regulates the dynamic instability of microtubules that allows reorganisation of the cytoskeleton (Feinstein and Wilson, 2005; Mandelkow and Mandelkow, 2012).

Besides regulating microtubule dynamics, tau regulates axonal transport by influencing the motor proteins dynein and kinesin, which transport cargoes towards the minus ends (towards the cell body) and plus ends of microtubules (towards the axon terminus), respectively (Fig 1.5). Tau can dynamically regulate the function of the axonal transport machinery through multiple mechanisms (Dixit et al., 2008; Ebner et al., 1998; Utton et al., 1997), which will be described in further detail below.

Table 1.2 Tau binding partners

Tau binding partner	Function of binding partner	References
ApoE3	Lipid carrier	(Fleming et al., 1996; Strittmatter et al., 1994)
β -Tubulin	Cytoskeleton	(Kar et al., 2003)
cSrc	Src-family kinase	(Lee et al., 1998; Reynolds et al., 2008)
F-actin	Cytoskeleton	(Fulga et al., 2007)
Fgr	Src-family kinase	(Reynolds et al., 2008)
Fyn	Src-family kinase	(Lee et al., 1998; Reynolds et al., 2008)
Grb2	Adaptor protein for growth factor signalling	(Reynolds et al., 2008)
Lck	Src-family kinase	(Lee et al., 1998)
p85 α	Regulatory subunit of PI3K	(Reynolds et al., 2008)

Phosphatidylinositol	Signalling lipid	(Surridge and Burns, 1994)
Phosphatidylinositol bisphosphate	Signalling lipid	(Flanagan et al., 1997)
PLC γ	Cleaves phospholipids into signalling molecules	(Hwang et al., 1996; Jenkins and Johnson, 1998; Reynolds et al., 2008)
PSD-95	A major postsynaptic scaffold protein at excitatory synapses	(Ittner et al., 2010; Mondragón-Rodríguez et al., 2012)

The table partially describes a number of proteins interacts with tau. ApoE3: Apolipoprotein E3; Grb2: Growth factor receptor-bound protein 2; PLC γ : Phosphoinositide phospholipase γ ; PI3K: Phosphoinositide 3-kinase; PSD-95: Postsynaptic density protein 95; Table modified from Morris et al., 2011 (Morris et al., 2011).

Tau can also serve as a direct inhibitor of histone deacetylase-6 (HDAC6), which deacetylates tubulin and may regulate microtubule stability (Perez et al., 2009) Thus, tau may affect microtubule stability by a mechanism independent of tubulin binding, However, reports regarding the levels of acetylated tubulin in tau knockout mice vary; some suggesting that acetylation is increased after tau deletion and others detect no change in acetylated tubulin, between tau knockout mice and wild-type controls (Perez et al., 2009; Rapoport et al., 2002).

As expected, increased acetylation was also found in tubulin from wild-type mice compared with tubulin from mice lacking tau because of the tau-mediated inhibition of the deacetylase.

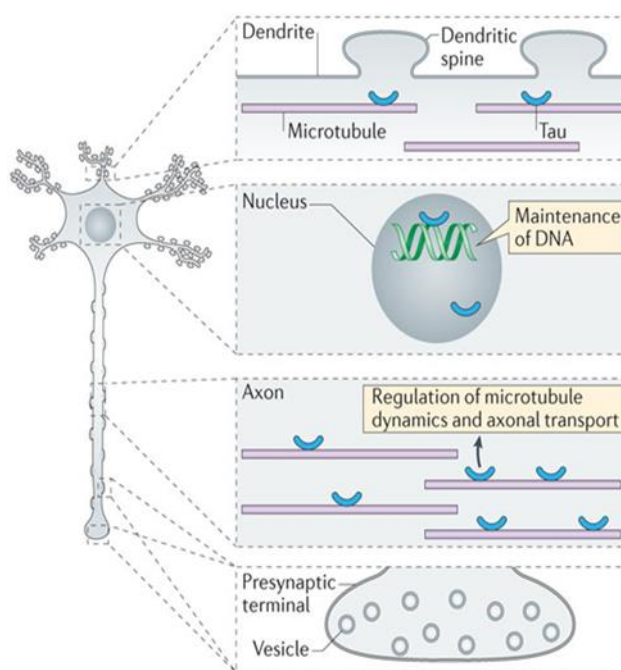
1.3.2 Signalling functions of tau

It is becoming increasingly clear that tau can act as a signalling scaffold, modulating the function of its binding partners via direct binding (Table 1.2).

In mouse brain, tau interacts with both the tyrosine kinase Fyn and the scaffolding protein Postsynaptic density protein 95 (PSD-95). Knocking out tau abolishes the translocation of Fyn into postsynaptic sites in dendrites (Ittner et al., 2010). This

finding raised the speculation that tau normally tethers Fyn to PSD-95/N-methyl-D-aspartate receptor (PSD-95/NMDA receptor) signalling complexes.

Figure 1.5 Physiological functions of tau



In healthy neurons, tau mainly distributes in axons and plays an important part in stabilising microtubules. Besides regulating microtubule dynamics, tau may regulate axonal transport through different mechanisms. Some tau also locates to membrane and in the nucleus as a protector for genomic DNA (Wang and Mandelkow, 2016).

Data from PC12 cells also indicated that tau is of importance in mediating nerve growth factor (NGF) signalling. Overexpression of 2N4R human tau was associated with normal neurite extension and an increased number of neurites per cell, whereas overexpression of the N-terminus of tau suppressed NGF-induced neurite extension (Brandt et al., 1995). Following up this notion, enhancement of NGF signalling by tau may involve increased association of tau with actin filaments, which occurs after stimulation with NGF and is mainly mediated by the microtubule binding domain rather than the N-terminus (Yu and Rasenick, 2006). Furthermore, also in PC12 cells, NGF and epidermal growth factor (EGF) mediated activation of mitogen-activated protein kinase (MAPK) is facilitated in the presence of tau (Leugers and

Lee, 2010). Furthermore, tau phosphorylation at Thr 231, is necessary for the growth factor-induced activation of the Ras-MAPK pathway (Leugers and Lee, 2010). Given that tau is not known to directly interact with growth factor receptors, it may facilitate signaling by binding to adaptor proteins such as Grb2 (Reynolds et al., 2008).

1.3.3 Functions of nuclear tau

Besides its role in regulating cytoskeleton dynamics and signalling pathways, tau has also been detected in the nuclei of both neurons and in non-neuronal cells, including human fibroblasts and human neuroblastoma cells (Loomis et al., 1990; Sjöberg et al., 2006). Nuclear tau appears to play a part in maintaining the integrity of genomic DNA, cytoplasmic RNA and nuclear RNA and hence may play a protective role in the nucleus (Sultan et al., 2011; Violet et al., 2014).

1.3.4 Tau and neuronal activity

Tau knockout mice have been a powerful tool in the search for novel functions of tau, although early investigations showed that young tau knockout mice present no overt phenotype (Harada et al., 1994). More recently, pathological changes observed in tau knockout mice have implied that tau may be involved in the regulation of neuronal activity, especially neurogenesis, and long-term depression (LTD). Recent evidence also suggests a role for tau in synaptic plasticity. A selective deficit in LTD but not in long-term potentiation (LTP) was observed in the corpora amylacea 1(CA1) region of the hippocampus in tau-knockout mice in vivo and ex vivo (Kimura et al., 2014). Adult hippocampal neurogenesis was severely impaired when the tau gene was knocked out from human tau transgenic mice (Hong et al., 2010). In contrast, knocking out tau from wild-type mice led to a decrease in migration of new-born neurons from the subgranular zone of the hippocampal formation to the granular layer, instead of a reduction in neurogenesis (Fuster-Matanzo et al., 2009). Although results acquired from these tau knockout mouse lines are inconsistent, both papers point to the possible involvement of tau in neurogenesis.

1.4 Pathological roles of tau

1.4.1 Tau oligomerisation and aggregation

One of the most prevailing ideas on how tau contributes to the pathogenesis of tauopathies is that tau undergoes misfolding and oligomerisation into insoluble tau

deposits, which gradually overburden neurons and ultimately cause neuronal cell death. Indeed, appearance of tau deposits has been regarded as a typical pathological signature in tauopathies, especially AD. The tauopathies are heterogeneous, with spatiotemporal differences in affected brain areas, but also in the splice variants of aggregated tau (Lee et al., 2001).

Tau is a natively unfolded protein (Jeganathan et al., 2008b; Schweers et al., 1994). Additionally, X-ray scattering, Fourier transform infrared spectroscopy, circular dichroism, and fluorescence spectroscopy also pointed to a localised folding which brings the N- and C-terminal domains to form a “paper clip” structure (Fig 1.3) (Jeganathan et al., 2008a). Despite the lack of ordered structure of full-length tau, there are two hexapeptide motifs located in the second and third microtubule-binding repeats that display high β -sheet propensity and are further characterised as drivers of abnormal self-assembly of tau (Mukrasch et al., 2009; Mukrasch et al., 2007). The hexapeptide motifs comprise residues 306–311 (PHF6, VQIVKY) and 317–335 (PHF6*: VQIINK) (Perez et al. 1996). These regions of tau self-assemble in the absence of any additional compounds (von Bergen et al. 2000; Santa-Maria et al. 2005). *In vitro* studies have demonstrated that PHF6 and PHF6* can form fibrillar aggregates in the presence of ammonium acetate (von Bergen et al., 2001). PHF6 is located at the beginning of the third MTB repeat and is present in all tau isoforms. In contrast, PHF6* is located at the beginning of the second MTB repeat and is present only in 4R tau isoforms since it is encoded by E10. Correspondingly, introduction of β -sheet disrupting amino acid substitutions, such as prolines, into the hexapeptide motifs, renders the protein incompetent for assembly (Bulic et al., 2010). Deletion of the positively charged K280 residue, which is involved in localised electrostatic interactions, hinders tau self-assembly (von Bergen et al., 2001). Conversely, as shown in Fig 1.6, mutations within the hexapeptide motif that enhance β -sheet propensity, such as the P301L mutation found in FTLD-tau, promotes tau aggregation (Lewis et al., 2000).

Formation of soluble tau monomers has been posited as the first step in detaching tau from microtubules and exceeding the cytosolic tau threshold concentration necessary to support aggregation (Golde, 2006). As summarised above, this dissociation could be mediated by abnormal phosphorylation (Liu et al., 2007), truncation (Binder et al., 2005) acetylation (Min et al., 2015) and other post-translational modification of tau. However, such changes also alter the properties of tau, such as electrostatic modification in the molecule, as well as exposure of

aggregation-competent motifs, eventually allowing the formation of tau–tau dimers (Andronesi et al., 2008). Tau dimerisation can emerge via intermolecular interactions between two PHF6, two PHF6* motifs, or between one PHF6 and one exogenous PHF6* motif (Peterson et al., 2008). Further recruitment of many other monomers and dimers of tau could lead to the formation of a ‘nucleation’ or aggregation centre (Barghorn and Mandelkow, 2002). Once a critical nucleus cluster size is reached, oligomerisation proceeds in a dose and time-dependent manner. Finally, tau oligomers elongate into subunits of filaments, termed protomers, which adopt the parallel, in register, cross β -sheet structure, typical of amyloid aggregates (Meraz-Rios et al., 2010). Ultimately, these tau filaments become the building blocks of NFTs.

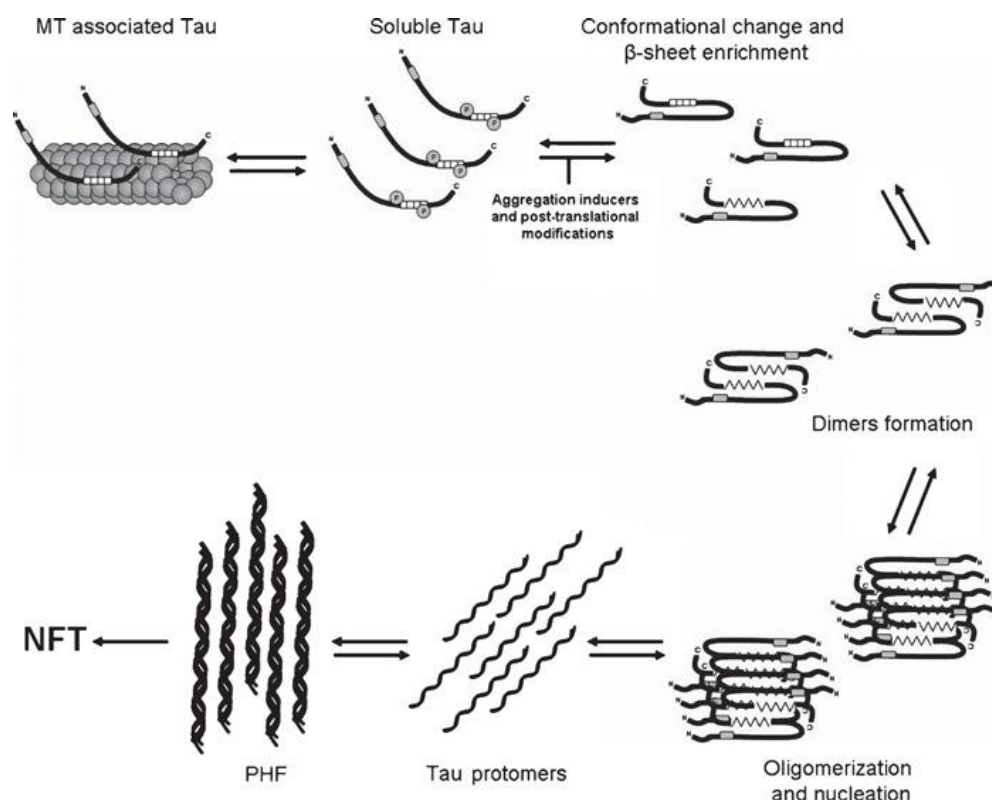
NFTs have long been considered to be toxic to neurons. However, contradictory findings have raised the need for further investigation to fill the gap between presence of NFTs and neurodegeneration. An *in vivo* model in which formaldehyde was used to treat primary hippocampal cells, showed that tau aggregates could induce apoptosis (Nie et al., 2007). The same toxicity was observed in a cell model that used N2a mouse neuroblastoma cells. Expression of a fragment of mutant K18 Δ K280 tau (comprising residues S258–I360, lacking K280) either alone, or together with full length mutant tau (Δ K280) caused cytotoxicity, and the cells were positive for thioflavin-S staining (Wang et al. 2007), implying NFTs are associated with cytotoxicity. In contrast, findings from a mouse strain expressing the same tau mutant in an inducible manner, demonstrated that memory is improved and neuronal loss is halted when the mutant tau gene is switched off, without changing NFT accumulation (SantaCruz et al., 2005). Whether NFTs *per se* are toxic is still unknown, however, it is clear that tau species generated during the formation of NFT are damaging to cells.

1.4.2 Tau and amyloid- β : from the “amyloid cascade hypothesis” to the “tau axis hypothesis”

In the early 1990s, in order to explain the interplay between A β -containing plaques and tau-containing NFTs in the onset and development of AD, the “amyloid cascade hypothesis” was proposed (Hardy and Higgins, 1992). This hypothesis postulated that formation of neuritic plaques stimulates subsequent pathological events, including the formation of NFTs and disruption of synaptic connections, leading to reduced neurotransmission, death of tangle-bearing neurons and dementia (Hardy

and Selkoe, 2002). Since then, extensive research using various models has further highlighted tau as a likely secondary effector of A β toxicity. In 2011, Ittner and Götz took this hypothesis a step further by suggesting three possible ways via which A β and tau proteins interact: (1) A β drives tau pathology; (2) synergistic toxic effects of A β and tau; and (3) tau may mediate A β toxicity (Ittner and Gotz, 2011).

Figure 1.6 Tau aggregation cascade



Abnormal post-translational modification of tau causes it to detach from microtubules (MT). Cytosolic tau forms dimers via interactions between β -sheet propensity motifs, providing nuclei for subsequent recruitment of more abnormal tau. Higher-order oligomers of tau gradually grow to protomers, and then tau filaments, which ultimately form neurofibrillary tangles (NFTs). In the case of AD, NFTs are composed of paired helical filaments (PHFs) (Meraz-Rios et al., 2010).

However, the amyloid cascade hypothesis has also been substantially challenged as data from tau knockout models has accumulated. As a result, the “tau axis hypothesis” has been put forward and is gaining increasing attention (Ittner and Gotz,

2011). The hypothesis suggests that increased tau in dendrites can make neurons more vulnerable to damage caused by A β at the postsynapse (Ittner and Gotz, 2011). Hippocampal neurons from tau knock-out mice are less sensitive to A β pathology (Ittner et al., 2010; Roberson et al., 2007). Genetic depletion of tau in cultured hippocampal neurons from wild-type mice showed that tau was required for pre-fibrillar A β -induced microtubule disassembly (Amadoro et al., 2011). Also, reduction of tau prevents A β -induced defects in axonal transport of mitochondria (Vossel et al., 2010). Taken together, these findings are shifting tau as an effector in the AD towards being a crucial partner of A β in disease pathogenesis.

1.4.3 Tau and α -synuclein

The presence of Lewy bodies formed of abnormally aggregated α -synuclein is the major neuropathological hallmark of Parkinson's disease (PD). It has been suggested that α -synuclein may also be involved in the pathogenesis of AD (Korff et al., 2013).

Correlation between tau and α -synuclein pathology has been demonstrated by the finding that phosphorylated tau is present in Lewy bodies (Merdes et al., 2003). Furthermore, α -synuclein binds to tau and primes it for phosphorylation by protein kinases (Hashiguchi and Hashiguchi, 2013). For example, α -synuclein stimulates tau phosphorylation by glycogen synthase kinase 3 beta (GSK3 β) through formation of a protein complex with these two proteins. Accumulation of α -synuclein induced by oxidative stress may lead to excessive phosphorylation of tau by GSK3 β (Kawakami et al., 2011). Other studies have reported that α -synuclein interacts directly with tau and stimulates its phosphorylation by cyclic AMP-dependent protein kinase (PKA) (Qureshi and Paudel, 2011). In the presence of α -synuclein, phosphorylation of tau at Ser262 is increased by PKA. In parallel, phosphorylation of tau at Ser262 causes tau to detach from microtubules, engendering microtubule instability, and making tau neurotoxic in *Drosophila* and in cultured primary neurons (Qureshi and Paudel, 2011; Whiteman et al., 2009). These results indicate that α -synuclein is a regulator of phosphorylation of tau at Ser262 and this may have a functional role in the maintenance of neuronal health.

1.4.4 Post-translational modifications of tau: linking physiology to pathology

Tau is subject to a complex array of post-translational modifications. So far, at least eleven different modifications have been identified (Martin et al., 2011).

Tau is modified by serine, threonine and tyrosine phosphorylation (Lee et al., 2004), isomerisation (Miyasaka et al., 2005), glycation (Ledesma et al., 1994), nitration (Reyes et al., 2008), O-GlcNAcylation (Shane Arnold et al., 1996), acetylation (Cohen et al., 2011), oxidation (Landino et al., 2004), polyamination (Wilhelmus et al., 2009), sumoylation (Dorval and Fraser, 2006), ubiquitination (Cripps et al., 2006) and proteolytic cleavage or truncation (Mondragón-Rodríguez et al., 2009). These modifications are highly interconnected and thus reach an equilibrium which maintains the normal function of tau. However, when such cooperation goes awry, pathological cascades may be triggered, potentially due to tau dysfunction and/or aberrant interactions with other proteins. Therefore, the crucial role of post-translational modifications of tau during its pathological transition is evident. Among the modifications mentioned above, the roles of tau phosphorylation, acetylation and truncation are particularly associated with disease progression in AD.

1.4.5 Tau phosphorylation

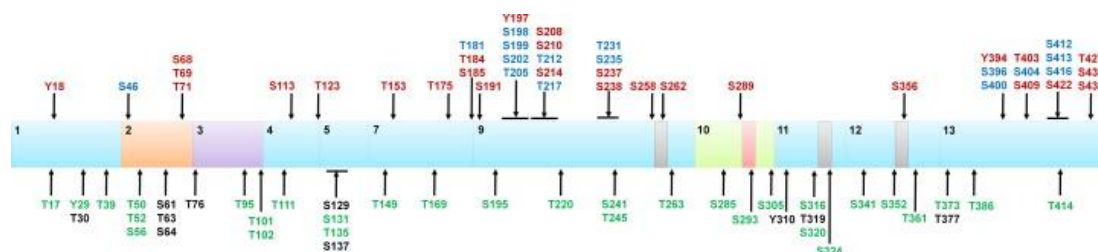
Tau phosphorylation in physiology and pathology

Phosphorylation is the most commonly described post-translational modification of tau. Tau contains 85 putative phosphorylation sites (Fig 1.7), including 45 serine (53% of phosphorylation sites of tau), 35 threonine (41%) and 5 tyrosine (6%) residues (Buée et al., 2000; Hanger et al., 2009; Sergeant et al., 2008).

Given its high number of potential phosphorylation sites, it is not surprising that phosphorylation has a profound impact on the physiological function of tau. Under pathological conditions, tau phosphorylation is increased, which results from an increase in the number of sites phosphorylated in the same tau molecule and/or an increase in the number of tau molecules phosphorylated at a given site. Increased tau phosphorylation reduces its affinity for microtubules, resulting in cytoskeleton destabilisation in neurons. Phosphorylation of tau at Ser262, Ser293, Ser324 and Ser356, respectively located in the first, second, third and fourth microtubule binding domains, have been shown to decrease tau binding to microtubules (Drewes et al., 1995; Sergeant et al., 2008). *In vitro* studies have shown that tau phosphorylation at

Thr214, Thr231 and Ser235 also contribute to the dissociation of tau from microtubules (Ksiezak-Reding et al., 2003; Sengupta et al., 1998) .

Figure 1.7 Tau phosphorylation sites



Tau phosphorylation sites found in AD brains (red), those found in control brain (green) and those present both in both control and AD brain (blue) are indicated according to the longest human tau isoform (2N4R). Putative phosphorylation sites that have not yet been validated are indicated in black. Figure uses single letter nomenclature for amino acids (Martin et al., 2011).

The link between abnormal phosphorylation and self-aggregation of tau has long been established (Braak and Braak, 1995; Iqbal and Grundke-Iqbal, 1991). Tau phosphorylation, probably at specific epitopes, decreases tau binding to microtubules and alters microtubule stability. The detached tau then undergoes self-aggregate (Fischer et al., 2009; Köpke et al., 1993; Von Bergen et al., 2000). Recent evidence has shown that pseudophosphorylated tau, which mimics permanent pathological tau phosphorylation by substitution of phosphorylatable residues with negatively charged glutamate or aspartate, reproduces structural and functional aspects of AD and exerts neurotoxic effect, including caspase activation and apoptosis initiation (Cho and Johnson, 2004b; Fath et al., 2002; Gong and Iqbal, 2008; Zhang et al., 2009). Tau phosphorylation in the proline-rich region disrupts its microtubule assembly activity and slightly promotes tau self-aggregation (Eidenmuller et al., 2001). Contrastingly, tau phosphorylation at the C-terminal tail region markedly promotes tau self-aggregation (Liu et al., 2007).

In addition to the classic loss-of-function and gain-of-toxicity paradigm, several lines of investigation have noted that increased tau phosphorylation may induce pathology through other mechanisms. Firstly, elevated phosphorylation of tau might

not only detach tau from microtubules, but could also subsequently induce tau mis-sorting from axons to the somatodendritic compartment, compromising axonal microtubule integrity and inducing synaptic dysfunction (Hoover et al., 2010; Thies and Mandelkow, 2007; Zempel et al., 2010). Second, phosphorylation of tau may disrupt its degradation. Tau phosphorylated at Ser262 or Ser356 is not recognised by the C-terminus of heat shock protein 70-interacting protein–heat shock protein 90 (CHIP–HSP90) complex and is thus protected from proteasomal degradation (Dickey et al., 2007). Phosphorylation of tau at Ser422 prevents its cleavage by caspase-3 at Asp421 in tau (Guillozet-Bongaarts et al., 2006). Finally, phosphorylation may change the association of tau with its interaction partners, disturbing the function of tau in a range of signalling pathways.

Tau kinases

Tau phosphorylation is tightly controlled by the balance between the activity of various protein kinases and phosphatases (De-Paula et al., 2010; Hanger et al., 2009; Yu et al., 2009). Tau kinases can be grouped into three broad classes: (1) proline-directed serine/threonine protein kinases, including GSK3 β , cyclin-dependent kinase-5 (Cdk5) and mitogen-activated protein kinases (MAPKs), which includes a wide range of kinases, including those activated by stress; (2) non-proline-directed serine/threonine protein kinases, such as tau-tubulin kinase1/2 (TTBK1/2), casein kinase 1 (CK1), dual-specificity tyrosine phosphorylation regulated kinase 1A (DYRK1A), microtubule affinity-regulating kinases (MARKs), Akt, cAMP-dependent protein kinase A (PKA), protein kinase C (PKC), protein kinase N and Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), and (3) protein kinases specific for tyrosine residues (Martin et al., 2011; Sergeant et al., 2008).

GSK3 was identified as a tau protein kinase in the 1990s, both isoforms of GSK3, GSK3 α and GSK3 β induce increased phosphorylation of tau (Hanger et al., 1992; Ishiguro et al., 1992; Ishiguro et al., 1993). Up to 42 putative phosphorylation sites in tau have been identified as targets of GSK3. Amongst these GSK3 target sites, 29 have been shown to be phosphorylated in AD brain (Hanger et al., 2009; Lovestone et al., 1994; Reynolds et al., 2000). Both the protein and activity level of GSK3 in tauopathy brain seems to correlate with the progress of neurodegeneration. Over-activation of GSK3 β significantly contributes to tau phosphorylation (Bitner et al., 2009; Greco et al., 2009; Hall et al., 2009; Nicolia et al., 2010; Qian et al., 2010). The post-mortem analysis of brains from AD patients and age-matched control samples indicates that the level of GSK3 is increased in neurodegeneration (Pei et

al., 1997) and the activity of GSK3 correlates with the increasing amount of NFTs (Leroy et al., 2002). Moreover, GSK3 β co-localizes with NFTs (Hanger et al., 1992; Yamaguchi et al., 1996). Moreover, tau phosphorylation by GSK3 β has been shown to induce tau aggregation (Rankin et al., 2007). GSK3 β phosphorylates tau at Thr231 and primes the C-terminus of tau for subsequent phosphorylation, promoting NFT formation (Cho and Johnson, 2004a; Rudrabhatla and Pant, 2010). In transgenic mice, inhibition of GSK3 β reduces tau phosphorylation (Caccamo et al., 2007; Engel et al., 2006; Nakashima et al., 2005), tauopathy and neurodegeneration (Noble et al., 2005), blocks NFT formation (Engel et al., 2006; Leroy et al., 2010) and rescues neuronal loss (Serenó et al., 2009). These data suggest that inhibition of GSK3 β is a promising therapeutic strategy that could be the most effective for AD, however positive results from clinical trials of GSK3 inhibitors are still elusive (Martin et al., 2009; Mazanetz and Fischer, 2007; Noble et al., 2005). Although GSK3 α has been shown to be a tau kinase, current research has focussed more on its role in regulating the production of A β peptides (Asuni et al., 2006; Lovestone et al., 1994; Phiel et al., 2003).

Importantly, the roles of other kinases, such as Cdk5, p38MAPK and PKA, in tau phosphorylation and NFT formation should not be excluded. An association between Cdk5, tau phosphorylation and NFT formation has been established in transgenic mice (Cruz et al., 2003; Noble et al., 2003). MAPKs also phosphorylate tau (Feijoo et al., 2005; Sergeant et al., 2008) and are detected in NFTs of AD brain (Zhu et al., 2000). CK1 Δ may also be considered to be a candidate tau kinase since it phosphorylates tau on 46 sites (Hanger et al., 2007; Li et al., 2004; Sergeant et al., 2008). Co-localisation of CK1 with tau aggregates has also been reported in AD brain (Kuret et al., 1997; Schwab et al., 2000). DYRK1A phosphorylates tau on Ser202, Thr212 and Ser404. Through T212 phosphorylation, this kinase could act as a priming kinase for subsequent tau phosphorylation by GSK3 β on Ser208 (Ryoo et al., 2007; Woods et al., 2001).

Tau is also phosphorylated on five tyrosine residues including Tyr18, Tyr29, Tyr197, Tyr310, Tyr395 (Derkinderen et al., 2005; Lebouvier et al., 2009; Sato et al., 2006). These sites are phosphorylated by Src family kinases, such as Src (Lee, 2005), Lck (Williamson et al., 2002), Syk (Lebouvier et al., 2008), Fyn (Lee et al., 2004) and c-Abl (Cancino et al., 2011; Derkinderen et al., 2005; Estrada et al., 2011). The tyrosine phosphorylation state of tau also seems to correlate with its propensity to aggregate (Hanger et al., 2009). Tyrosine-phosphorylated tau at Y18 was detected

in both soluble and sarkosyl-insoluble preparations derived from brain and spinal cord, and localized in neurons containing aggregated tau in mice which expresses human tau with P301L mutation (Vega et al., 2005). Interestingly, Y18 phosphorylation seems to play different role in different tauopathies. In mice expressing P301L tau, phosphorylation at Y18 of tau concurrently occurs with AT8 reactivity, which is an established marker of tau pathology (Bhaskar et al., 2010). In contrast, this correspondence sometimes is missing in 3×Tg-AD mice and AD brains, both of which manifested A β and tau pathology, implying different roles of tau tyrosine phosphorylation in these disease (Bhaskar et al., 2010; Lee et al., 2004). Supporting this assumption is the finding that in APP23 mice overloaded with high A β , tau mediated A β -induced excitotoxicity via its interaction with Fyn, a tyrosine kinase of tau (Ittner et al., 2010). Moreover, phosphorylation at Y18 of tau is required for A β induced cell cycle re-entry, another pathological effect leading to neuronal cell death (Seward et al., 2012).

Tau phosphatases

Protein phosphatase 2A (PP2A) is the major protein phosphatase in the brain that dephosphorylates tau at several phosphorylation sites (Liu et al., 2005a). PP2A accounts for more than 70% of cellular phosphatase activity (Liu et al., 2005a) and is implicated in the regulation of tau phosphorylation (Gong et al., 2000; Gong et al., 1993; Kuszczuk et al., 2009; Nuydens et al., 2000; Virshup and Shenolikar, 2009; Xu et al., 2008). *In vitro*, co-incubation of misfolded tau isolated from AD brains with PP2A restores tau binding to microtubules to a level similar to that recombinant human brain 2N4R tau (Wang et al., 2007).

PP2A activity has been shown to be decreased by approximately 50% in AD brains (Gong et al., 1993; Liu et al., 2005a; Rahman et al., 2005). Reductions in PP2A activity can result from the inhibitory effect of the PP2A catalytic subunit after phosphorylation at Y307 (Liu et al., 2008), or from increased activity of endogenous inhibitors of PP2A (Chen et al., 2008; Saito et al., 1995; Tanimukai et al., 2005). Similarly, protein phosphatase 5 (PP5) also dephosphorylates tau and tau aggregates isolated from AD brain (Gong et al., 2004; Liu et al., 2005b). In AD brain, the enzymatic activity of PP5 is also reduced by 20% (Liu et al., 2005b). On the contrary, overexpression of PP5 protects neurons against apoptosis induced by A β (Sanchez-Ortiz et al., 2009). It is worth mentioning that, in some cases, PP2A activity may override the protein kinase activities of GSK3 β and Cdk5 (Planel et al.,

2001). These data indicate that down-regulation of dephosphorylation or excessive activation of phosphorylation of tau is implicated in the formation of tau pathology.

Importantly, Akt plays a critical role in maintaining the balance between the actions of GSK3 β and PP2A by directly governing the activity of GSK3 β . Upon phosphorylation by phosphoinositide 3-kinase (PI3K) on Ser473, Akt inactivates GSK3 α and GSK3 β through phosphorylating GSK3 α/β on Ser21/9 respectively (Partovian and Simons, 2004). Consequently, inhibition of PI3K/Akt signalling pathway causes GSK3 β activity to increase and leads to increased tau phosphorylation, and thus NFT formation (Lee et al., 2003). Accordingly, a PI3K inhibitor (such as wortmannin) increases tau phosphorylation (Deng et al., 2005; Liu and Wang, 2002). In addition, PI3K/Akt signalling is attenuated in the brains of patients with AD (Mercado-Gomez et al., 2008).

The mammalian target of rapamycin (mTOR) also regulates the activity of PP2A, and inhibition of mTOR activates PP2A (Bishop et al., 2006). PP2A has been reported to dephosphorylate GSK3 β at Ser9 (Lee et al., 2005). In contrast, activation of GSK3 β can inhibit PP2A (Yao et al., 2011). These results suggest a regulatory loop between PP2A and GSK3 β to maintain the phosphorylation status of tau. When Akt/mTOR signalling is affected, this loop is also perturbed, yielding subsequent damaging consequences (Meske et al., 2008).

1.4.6 Tau truncation

Tau fragmentation in disease and in disease-relevant models

Proteolytic cleavage of disease-modifying proteins is found in a wide variety of human neurodegenerative diseases, including AD (Gamblin et al., 2003; Guo et al., 2004; Horowitz et al., 2004; Nieto et al., 1991; Novák, 1994; Novak et al., 1993; Rissman et al., 2004; Wischik et al., 1988a; Wischik et al., 1988b), PiD (Guillozet-Bongaarts et al., 2007; Mondragón-Rodríguez et al., 2008), CBD and PSP (Arai et al., 2004), Transactive response DNA-binding protein 43 (TDP-43) related FTLD (Igaz et al., 2009; Igaz et al., 2008), and PD (Anderson et al., 2006), as well as polyglutamine diseases, such as Huntington's disease (HD) (Gafni and Ellerby, 2002; Gafni et al., 2004).

Goedert and colleagues, using an antibody against the highly purified protease-resistant PHF core, provided the initial evidence that cleaved tau is actively involved in tau aggregation and the formation of PHFs (Goedert et al., 1988). These findings

revealed that the PHF core consists of tau fragments of 12 and 9.5 kDa. This finding was further supported by the discovery a 33 kDa N-terminally truncated tau from PHFs purified from human AD brain (Nieto et al., 1991). Novak and colleagues reported that the same antibody recognised only tau protein that was C-terminally truncated at Glu391 (Novak et al., 1993). This provided the first *in situ* evidence to suggest that tau truncation is a part of the pathological process of tau protein misfolding in AD. Similar results were also gained from immunohistochemical analysis of brain tissues from AD patients of different age and gender (Bondareff et al., 1990).

A tau fragment cleaved at Asp421 by activated caspase-3 has also been identified *in vitro* and in transfected COS and NTera-2 (NT2) cells (Fasulo et al., 2000). The presence of these caspase-cleaved products of tau in AD brain was confirmed by two antibodies specific for caspase-cleaved tau, namely TauC3 and α - Δ Tau (Gamblin et al., 2003; Rissman et al., 2004). Notably, cognitive decline and formation of NFTs in aged wild-type mice is also significantly correlated with increased caspase activity and tau truncated by caspase-3 (Means et al., 2016). Application of the TauC3 antibody also revealed consistent labelling within NFTs, dystrophic neurites within plaque-rich regions, and the CA region in the hippocampus of human vascular dementia brain. In addition, active caspase-3 has been demonstrated to co-localise within plaques, blood vessels and pre-tangle neurons, with TauC3 (Day et al., 2015). Similarly, in AD brain, caspase-6-cleaved tau fragments are also associated with both early and late NFTs and these appear to correlate well with cognitive decline (Gamblin et al., 2003; Guo et al., 2004; Horowitz et al., 2004).

Recently, several lines of investigation have not only identified new tau fragments in cases of tauopathy, but have also indicated novel roles for tau truncation in the progression of a variety of neurodegenerative diseases. A 17 kDa tau fragment (Tau₇₃₋₃₁₅) was identified in cerebellar granule neurons undergoing apoptosis as a result of caspase-3 and calpain-mediated cleavage (Canu et al., 1998). Interestingly another 17 kDa tau fragment (Tau₄₅₋₂₃₀) was found in hippocampal neurons treated with A β (Park and Ferreira, 2005). Moreover, in human SH-SY5Y cells undergoing apoptosis as well as in the hippocampus of 15 month-old AD11 transgenic mice (a model exhibiting chronic NGF deprivation during adulthood and AD-like pathology, including A β accumulation and hippocampal-dependent memory deficits) an N-terminal neurotoxic tau fragment (Tau₂₆₋₂₃₀), namely NH2-tau fragment, was

detected (Corsetti et al., 2008). Wray and colleagues (Wray et al., 2008) described a 35 kDa C-terminal tau fragment (Tau₁₈₇₋₄₄₁) lacking the N-terminus of tau, but containing all four microtubule-binding repeats (4R), and present only in neurodegenerative disorders characterised by over-expression of 4R tau isoforms, particularly in PSP. Further studies showed that minimal expression of this tau fragment in transgenic mice (Tau35 mice) is sufficient to cause several key features of human tauopathy (Bondulich et al., 2016). Thus, Tau35 mice exhibit aggregates formed of abnormally phosphorylated tau, progressive cognitive and motor deficits and loss of synaptic components (Bondulich et al., 2016). Similarly, another C-terminal tau fragment (Tau₂₄₃₋₄₄₁), namely Tau-CTF24, was detected in another mouse model of 4R tauopathy (Tg601) (Matsumoto et al., 2015). This model exhibited synapse loss in the nucleus accumbens and axonopathy in the ventral medial prefrontal cortex as well as increased tau phosphorylation at the PHF-1 epitope in the striatum (Kambe et al., 2011).

Interestingly, a tau fragment (Tau₄₅₋₂₃₀), accumulates in lumbar and cervical spinal cord, as well as in upper motor neurons located in the precentral gyrus from Amyotrophic lateral sclerosis (ALS) subjects (Vintilescu et al., 2016). These findings suggest that in addition to AD and related tauopathies, tau fragmentation may have an important role in the mechanisms leading to degeneration of motor neurons in ALS.

How tau truncation might lead to the development of tau pathology

Tau cleavage could either generate fragments with a toxic gain-of-function, thereby switching on the cell death cascade, or alternatively it could induce and drive aggregation of tau and any associated disease-modifying proteins. Supporting the latter scenario, is the fact that truncated protein fragments are upstream in the proteopathic cascade in neurodegenerative diseases and these can form the initial seeds required for aggregation (De Calignon et al., 2010; Filipcik et al., 2012; Graham et al., 2006; Igaz et al., 2009; Levin et al., 2009; Zilka et al., 2006).

The protease resistance of the 12 kDa tau fragment identified in the core of PHFs, led to the suggestion that truncation may be the mechanism that transforms tau into a modified species that is prone to misfolding, adopting an abnormal conformation and self-assembling into PHFs (Novák, 1994). This view is supported in a study using DC11, a novel type of truncation-dependent conformational antibody, which recognises abnormal tau in AD brain, but not tau in non-demented control brain

(Vechterova et al., 2003) Recombinant tau proteins truncated either at the N-terminus or at both the N- and C-termini, are also recognized by DC11, indicating N- and C-terminally truncated tau species adopted pathological conformations (Vechterova et al., 2003). Similarly, *in vitro* studies of tau aggregation have indicated that truncations occurring at Glu391 and Asp421, produce tau proteins that are more prone to aggregation than is full-length tau (Abraha et al., 2000; Berry et al., 2003). The expression of Tau_{151–391} fragment in the brain of transgenic rats induced neurofibrillary degeneration similar to that found in human disease. Pathological features include increased tau phosphorylation, formation of Gallyas-positive intracellular and extracellular NFTs, with Congo Red birefringence and thioflavin S reactivity, and sarkosyl-insoluble tau containing both truncated and endogenous rat tau (Filipcik et al., 2012; Graham et al., 2006). Tau truncated at Asp421 co-localises with NFTs in AD brain as well as in mouse models of AD, indicating that the generation of this tau fragment may be an early event in NFT formation (Basurto-Islas et al., 2008; Gamblin et al., 2003; Horowitz et al., 2004). Recently, De Calignon and colleagues (De Calignon et al., 2010) have shown that transient activation of executioner caspases in neurons of Tg4510 transgenic mice, which inducibly express human P301L tau, led to tau cleavage at Asp421, generating tau fragments possessing tangle-related conformational epitopes, and thioflavin S-positive NFTs. These data, both *in vitro* and *in vivo* confirm that truncation causes conformational changes in tau that may be responsible for induction of a misfolding cascade that generates NFTs in the brain in AD and related tauopathies.

Tau truncation can also exert intracellular toxic effects via other mechanisms. In cultured neurons, A β treatment induces the formation of tau truncated at Asp421. These findings have led to the proposal that extracellular A β induces caspase activation and subsequent generation of tau truncated at Asp421, providing building blocks for tau filament formation (Gamblin et al., 2003; Rissman et al., 2004). However, subsequent studies have shown that this cascade involves other players. In Chinese hamster ovary cells (CHO), overexpression of a tau fragment comprising residues 1-421, did not result in tau aggregation unless it was co-expressed with GSK3 β (Cho and Johnson, 2004a). Moreover, pseudophosphorylation of Ser422 on tau can inhibit its truncation at Asp421 by caspase-3 *in vitro* (Guillozet-Bongaarts et al., 2006). Thus, the interplay between tau phosphorylation and truncation may actively modulating aggregation of tau.

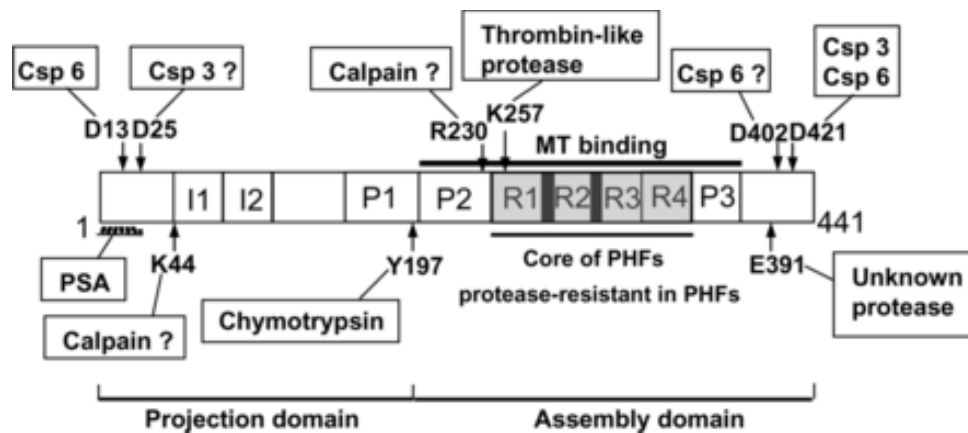
Expression of a truncated tau molecule comprising residues 151-421 in hippocampal neurons, led to the induction of apoptosis, suggesting that caspase cleavage of tau at Asp421 might convert it into an apoptosis effector molecule (Fasulo et al., 2000). It has also been shown that this truncated tau species induces mitochondrial fragmentation and elevates oxidative stress, compared with cells expressing full-length tau (Cente et al., 2006; Quintanilla et al., 2009). Another amino terminal tau fragment (residues 26–230) is enriched in human mitochondria in preparations of synaptosomes from AD, which correlates with the altered function and quality control of mitochondria at synapses and with synaptic dysfunction (Amadoro et al., 2010; Corsetti et al., 2015).

Recent data has also linked tau fragmentation with tau propagation between neurons. Thus, C-terminal truncated tau is abundant in synaptic terminals in aged control and in AD brain (Sokolow et al., 2015). Moreover, following depolarisation, tau release is potentiated in AD nerve terminals compared to aged controls, indicating that tau cleavage may facilitate tau secretion and propagation from the pre-synaptic compartment in AD. Upon uptake of extracellular seeds, tau fragments are more prone to aggregate. In a model related to Tau-CTF24 fragment (Tau₂₄₃₋₄₄₁), when insoluble tau seed was introduced, insoluble tau deposits formed more readily in SH-SY5Y cells overexpressing the Tau-CTF24 fragment compared to those overexpressing full-length tau (Matsumoto et al., 2015). Cell lysates containing the Tau-CTF24 inclusions propagated to naive tau-expressing cells more efficiently than did cells containing inclusions generated from full-length tau. Furthermore, aggregated Tau-CTF24 bound to cells more rapidly and abundantly than aggregated full-length tau as revealed by immunoblot and confocal microscopic analyses (Matsumoto et al., 2015). Taken together, these data suggest that tau truncation contributes to neurodegeneration by enhancing its prion-like propagation.

Mechanisms underlying tau truncation

Alongside the increasing number of tau fragments present in cell and animal models of disease, increasing numbers of proteases responsible for tau truncation have also been identified. Amongst these enzymes, caspases and calpain have been investigated most intensively and are described in more detail below. Additionally, recent studies have also proposed a number of other proteases that may play a role in tau cleavage, including thrombin, cathepsins, asparagine endopeptidase (AEP), puromycin-sensitive aminopeptidase (PSA) and proteasome (Fig 1.8) (Hanger and Wray, 2010; Wang et al., 2010; Zhang et al., 2014b) .

Figure 1.8 Diagram of proteolytic cleavage sites on tau



Tau is cleaved by chymotrypsin after Tyr197, resulting in an N-terminal projection domain and a C-terminal assembly domain, which contains the microtubule-binding elements (Steiner et al., 1990). The repeat domain of tau (R1–R4) represents the core of PHFs and is highly protease-resistant in PHFs (Von Bergen et al., 2006; Wischik et al., 1988a). The MT-binding region of tau contains the repeat domains plus the flanking proline-rich domains P2 and P3. Cleavage sites after Asp13 by caspase (Csp) 6 (Horowitz et al., 2004), Asp421 by caspase 3 (Gamblin et al., 2003; Rissman et al., 2004), and Lys257 by an unknown thrombin-like cytosolic protease (Khlistunova et al., 2006; Wang et al., 2007) were validated by N-terminal protein sequencing or mass spectrometry. Cleavage after Asp25 (putative caspase 3 cleavage site) (Rohn et al., 2002), Asp402 (putative caspase 6 cleavage site) (Guo et al., 2004) and Glu391 was determined with site-directed antibodies. Glu391 is cleaved by an unknown protease (Novak et al., 1993; Wischik et al., 1988b). Cleavage after Lys44 and Arg230 was proposed from putative calpain cleavage sites found on tau according to the P2-P1 rule (Park and Ferreira, 2005). Puromycin-sensitive aminopeptidase (PSA) removes residues stepwise from the N-terminus of tau (Karsten et al., 2006). Figure adapted from Wang et al., 2010 (Wang et al., 2010).

Caspases

Caspases belong to the cysteine protease family, which plays essential roles in apoptosis. All caspases are activated through proteolysis of procaspase zymogens (Shi, 2002). Caspases recognise on their substrates at least four contiguous amino acids, which are upstream of the enzyme cleavage site, named P4-P3-P2-P1. Caspases also have an absolute requirement for an aspartate residue in the P1 position before the scissile bond (Cohen, 1997). However, most caspases are

promiscuous at the P2 and P3 residues (Nicholson, 1999), thus the value of predictions of consensus sites for caspase cleavage is rather limited.

Data from *in vitro* experiments has shown that Asp421 in tau is targeted by caspases-1, -3, -6, -7 and -8, generating a fragment ~5 kDa smaller than full-length tau, due to removal of the C-terminus (Gamblin et al., 2003; Rissman et al., 2004). In addition, tau is also cleaved by caspase-6 *in vitro* at Asp13, even more efficiently than the cleavage at Asp421, and these cleavage sites have both been validated by N-terminal protein sequencing or/and mass spectrometry (Gamblin et al., 2003; Horowitz et al., 2004; Rissman et al., 2004).

Through the use of site-directed antibodies, truncation of tau at Asp25 (a putative caspase-3 cleavage site) and Asp402 (a putative caspase-6 cleavage site) has been observed in AD brain or transgenic animals, respectively (Guo et al., 2004; Rohn et al., 2002). However, to date, contradictory results have shown that these sites cannot be cleaved by any known caspases *in vitro* (Gamblin et al., 2003). Although activated caspase-6 was found to co-localise with tau aggregates in AD brain, direct evidence of tau truncation at Asp13 in AD remains elusive (Guo et al., 2004). Thus, only tau truncation at Asp421 has so far been validated both *in vitro* and *in vivo* and has been shown to be directly related to the development of tau pathology. Recently, the NH2-tau fragment, extensively reported to elicit neurotoxic effects in primary neuronal cultures, as well as mitochondria disarrangement, has also been found to be a product of caspase-cleavage that is generated during apoptosis (Amadoro et al., 2010; Corsetti et al., 2008; Corsetti et al., 2015).

Notably, pseudophosphorylation at Ser422 can abolish truncation of tau at Asp421 by caspase-3 *in vitro*, and phosphorylation at Ser422 in AD precedes truncation at Asp421 during NFT maturation indicating tau phosphorylation on Ser422 may inhibit tau cleavage by caspase *in vivo* (Guillozet-Bongaarts et al., 2006).

The role of a novel pro-apoptotic protein, appoptosin, has recently been proposed in neurological disorders with tau neuropathology, providing clues for understanding the upstream mechanism underlying caspase-mediated tau truncation (Zhang et al., 2012b). The physiological function of appoptosin is to transport/exchange glycine/5-amino-levulinic acid across the mitochondrial membrane for heme synthesis. Increased appoptosin and caspase-3-cleaved tau was observed in AD brain and FTLD-tau. Moreover, in tau transgenic mice, overexpression of appoptosin

increased caspase-mediated tau cleavage, tau aggregation, and synaptic dysfunction, whereas appoptosin deficiency reduced tau cleavage and aggregation (Zhao et al., 2015).

Calpains

Calpains are cytosolic Ca^{2+} -activated cysteine proteases. Calpain-1 and calpain-2, also known as μ -calpain and m-calpain, are the two major forms (Goll et al., 1992). Besides Ca^{2+} , calpain activity is negatively regulated by a Ca^{2+} -dependent heat-stable inhibitor, called calpastatin. Early investigations proposed that cleavage by calpains follows a P2-P1 rule, which states that the preferred residues on calpain substrates are leucine or valine at P2, and arginine or lysine at P1, just before the scissile bond (Sasaki et al., 1984). However, it is now well established that protein cleavages by calpains are only poorly related to amino acid sequence, and is more closely associated with the conformation of the polypeptide chain (Cuerrier et al., 2005; Tompa et al., 2004).

In AD brain, increased calpain activity is observed in comparison with age-matched controls (Saito et al., 1993) and calpastatin is depleted (Rao et al., 2008). Several studies have shown that tau can be degraded by calpains *in vitro* (Johnson et al., 1989). Pre-aggregated A β treatment of cultured neurons led to activation of calpain and production of Tau₄₅₋₂₃₀ (Park and Ferreira, 2005), which was also found in transgenic AD animal models exhibiting neurodegeneration (Roberson et al., 2007). The amino acid sequence of Tau₄₅₋₂₃₀ is confirmed by combining the potential calpain cleavage sites on tau and the apparent molecular mass (Hanger and Wray, 2010; Wang et al., 2010). Overexpression of Tau₄₅₋₂₃₀ induced apoptosis in both CHO cells and cultured neurons, and thus was proposed to have inherent neurotoxic properties (Park and Ferreira, 2005). However, the absence of the 17 kDa calpain-induced fragment in AD brain raises the need for further investigation into the relationship between this fragment and neurodegeneration in AD.

As is the case with caspases, the susceptibility of tau to cleavage by calpain is retarded by tau phosphorylation. Highly phosphorylated insoluble human brain tau is less susceptible to calpain degradation than is soluble tau, which has a lower phosphorylation state (Litersky and Johnson, 1992; Mercken et al., 1995), suggesting that phosphorylation may be linked to tau cleavage *in vivo*. However, calpain-mediated tau cleavage may also be hampered by the conformation adopted by insoluble tau during its deposition in the brain in AD.

Thrombin

Thrombin is an extracellular serine protease generated by proteolytic cleavage of its precursor, prothrombin, which is made primarily in the liver and circulates in plasma (Fenton, 1986). Thrombin has also been reported to be present in NFTs in AD (Akiyama et al., 1992; Arai et al., 2006), implying that it may be related to tau aggregation. The presence of prothrombin mRNA has been demonstrated in the olfactory bulb, the cortex, the cerebellum, and other regions of the rat and human nervous system, as well as in neural cell lines (Dihanich et al., 1991). Both prothrombin and thrombin have also been shown to be expressed in neurons using immunohistochemistry (Arai et al., 2006), and it was therefore proposed that thrombin is a protease that could proteolyse endogenous tau in the brain. Supporting this proposal is the finding that, in brain lysates incubated with different protease inhibitors, specific inhibition of thrombin was able to completely repress tau degradation (Arai et al., 2005). However, questions remain regarding the mechanisms underlying the proteolysis of prothrombin to thrombin in neurons and the existence of active thrombin within the cytoplasm.

In vitro, tau can be proteolysed by thrombin in a stepwise manner at multiple arginine and lysine sites including Arg155, Arg209, Arg230, Lys257 and Lys340. The initial cleavage occurs at Arg155, producing a tau fragment of 37 kDa (Wang et al., 2007). This truncated tau polypeptide is then cleaved further at Arg230, yielding a 25 kDa tau fragment derived from the C-terminal of tau and displaying a reduced capacity to promote microtubule assembly compared with full-length tau (Olesen, 1994).

Similar to other enzymes that cleave tau, phosphorylation of tau appears to make it more resistant to thrombin cleavage. Thrombin cleavage at Arg209, Arg230, Lys257 and Lys340 is suppressed by phosphorylation at Thr212, Thr231 and Ser396/Ser404 induced by GSK3 β (Arai et al., 2005). On the contrary, insoluble aggregated tau from AD brain becomes more susceptible to thrombin degradation following treatment with alkaline phosphatase to remove phosphate. Additionally, phosphorylation of tau by PKA also induces resistance to thrombin cleavage (Wang et al., 1996b), supporting the view that phosphorylation may be a mechanism that dynamically modulates proteolysis of tau.

Cathepsins

Cathepsins are a group of proteases which are normally located within lysosomes. Most cathepsins are cysteine proteases, with the exception of cathepsins A and G, which are serine proteases, and cathepsins D and E, which are aspartyl proteases.

Under pathological conditions, certain cathepsins can be released into the cytosol (Yang et al., 1998). Active cathepsin D and cathepsin B have been found in amyloid plaques in AD brain (Cataldo et al., 1990). Several groups have shown that tau is a substrate of cathepsin D *in vitro*, which cleaves tau between amino acids 200 and 257, resulting in the generation of a 29 kDa tau species (Bednarski and Lynch, 1996; Kenessey et al., 1997). In human neuroblastoma cells inducibly expressing tau, disruption of lysosomes with chloroquine results in inhibition of tau degradation and the appearance of tau aggregates (Wang et al., 2009). This study also indicated the involvement of cathepsins B and L, consistent with the other reports. In a cell model expressing a synthesised tau fragment, tau_{RD}ΔK280 (microtubule binding repeats with ΔK280 mutation), cathepsin L generated two amyloidogenic tau fragments (F2 and F3), thereby playing a role in tau aggregation (Hamano et al., 2008; Wang et al., 2009).

In contrast with the degradation of tau by calpain, caspase-3 or thrombin, where phosphorylation of tau suppresses proteolysis, tau degradation by cathepsin D appears to be accelerated by enhanced phosphorylation, at least of the foetal (0N3R) tau isoform *in vitro* (Kenessey et al., 1997).

As cathepsins are lysosomal proteases, an important question is how these enzymes could gain access to tau in neurons. One possible mechanism is by inefficient translocation of tau or tau fragments across the lysosomal membrane, leading to incomplete lysosomal cleavage of tau, which would generate small tau fragments (Wang et al., 2009). In AD and other conditions of cellular stress, cathepsin D and others proteases might contribute to tau proteolysis once the lysosomal system is disturbed (Anderson et al., 2000; Bondulich et al., 2016).

Asparagine endopeptidase

Recently, another lysosomal cysteine proteinase, asparagine endopeptidase (AEP) has emerged as a tau protease. AEP proteolytically degrades tau, abolishes its microtubule assembly function, and induces tau aggregation. Notably, AEP is upregulated in human AD brain and also in P301S tau transgenic mice. Knock down

of the AEP gene in P301S mice results in substantially reduced tau phosphorylation, rescue of hippocampal synaptic function impairment and also reduced cognitive deficits (Zhang et al., 2014b). Furthermore, introduction of tau N255A/N368A mutant, which does not process the cleaved site of AEP, also attenuated pathological and behavioural defects in the P301S mice.

Puromycin-sensitive aminopeptidase

PSA is a protease that belongs to the M1 class of metallopeptidases (Rawlings and Barrett, 1993). PSA is found in neurons, but not in surrounding glial cells or in blood vessels (Tobler et al., 1997). PSA comprises over 90% of the aminopeptidase activity in the brain (McLellan et al., 1988). PSA can digest recombinant tau as well as tau purified from brain tissue *in vitro*, thus PSA may suppress tau pathology through modulating tau levels. (Karsten et al., 2006; Sengupta et al., 2006). Correspondingly, expression of PSA is found to be inversely correlated with vulnerability to tau pathology (Karsten et al., 2006). In a *Drosophila* model of AD, PSA expression reduced tau and protected against tau-induced neurodegeneration, whereas flies expressing a PSA loss-of-function mutant exhibited exacerbated neurodegeneration. Interestingly, in FTLD-tau brain tissue, expression of PSA is elevated 5-fold in the cerebellum compared with the frontal cortex. This finding, combined with the observation that the cerebellum, is less affected than cerebral cortex in disease conditions such as AD and FTLD-tau (Ciavardelli et al., 2010; Karsten et al., 2006), further reinforce the role of PSA being protective against neurodegeneration

The ubiquitin-proteasome system

The ubiquitin-proteasome system (UPS) is a major cellular mechanism that regulates intracellular protein levels and eliminates damaged, misfolded, and mutant proteins for protein quality control in both the cytoplasm and the nucleus (Benaroudj et al., 2001; Ciechanover, 2001; Kisselev et al., 1998; Kisselev et al., 1999; Tofaris et al., 2001; Touitou et al., 2001). Blocking the activity of the 20S proteasome catalytic core inhibits tau degradation in transfected SH-SY5Y cells (David et al., 2002). Furthermore, *in vitro* experiments have shown that the 20S proteasome directly degrades unfolded recombinant tau in an ubiquitin-independent manner, generating stable tau intermediates (~27 and 17 kDa tau fragments) (David et al., 2002; Wang and Mandelkow, 2012). In AD brain, proteasome activity is decreased, which could contribute to the accumulation of protein aggregates, including tau filaments (Keller et al., 2000; Salon et al., 2000).

1.4.7 Tau acetylation

In addition to phosphorylation, acetylation of tau is also emerging as an important post-translational modification related to both normal and pathological functions of tau (Wang and Mandelkow, 2016).

The impact of tau acetylation can be either beneficial or detrimental depending on the target site. Acetylation at Lys259, Lys290, Lys321 or Lys353 occurs in normal tau, but is reduced in the brains of individuals with AD and in rTg4510 transgenic mice, which over-express P301L tau (Cook et al., 2014). Furthermore, acetylation at Lys259, Lys290, Lys321 or Lys353 protects tau from phosphorylation and suppresses further tau aggregation. By contrast, acetylation of tau at Lys280 has been detected in AD and other tauopathies, including PiD, FTLT-tau and PSP, and is considered to be pathological in these conditions (Irwin et al., 2013). This finding is supported by the observation that acetylation at Lys163, Lys280, Lys281 or Lys369 inhibits tau degradation through proteasome-mediated degradation and leads to accumulation of highly phosphorylated tau (Min et al., 2010). Recently, tau acetylation at Lys174 was also identified in human AD brain (Min et al., 2015). Acetylation of tau at this site appears to retard tau turnover and may be critical for tau-induced toxicity, suggesting that the acetylation of tau could represent a new therapeutic target for treatment of human tauopathies.

Regarding the enzymes that are responsible for acetylating tau, to date, P300 acetyltransferase and cAMP-response element binding protein (CREB)-binding protein have been found to acetylate tau at several Lys residues in the flanking region or the repeat domain of the polypeptide (Kamah et al., 2014; Min et al., 2010). Sirtuin 1 (SIRT1) and HDAC6 are responsible for tau deacetylation (Cook et al., 2014; Min et al., 2015). Notably, tau has intrinsic acetyltransferase activity and so it can catalyse auto-acetylation at certain Lys sites, including Lys280 (Cohen et al., 2013).

1.4.8 Other modifications of tau

In human AD brain, but not in control brain, tau is modified by glycosylation, which is proposed to help to maintain PHF structure (Wang et al., 1996a). Furthermore, glycosylation may facilitate tau phosphorylation, as it suppresses dephosphorylation and accelerates phosphorylation of tau, most likely by changing the conformation of tau (Liu et al., 2002). In contrast to N-glycosylation, O-GlcNAcylation (a type of O-

glycosylation) of tau may protect it from phosphorylation, as it competes with tau kinases to modify the same serine/threonine residues (Liu et al., 2004). In addition, O-GlcNAcylation of tau can suppress tau aggregation (Yuzwa et al., 2014). In AD, O-GlcNAcylation of tau is reduced, an effect that might contribute to the increased phosphorylation and aggregation of tau (Liu et al., 2004).

Non-enzymatic post-translational modifications, including glycation, deamidation and isomerisation, have also been detected in PHF-tau but not in tau from control brain (Watanabe et al., 2004; Yan et al., 1995). All of these modifications may facilitate tau aggregation (Ledesma et al., 1995; Watanabe et al., 2004). Furthermore, glycation of tau may reduce the binding of tau to microtubules (Reyes et al., 2012). Abnormal nitration of Tyr18, Tyr29 and Tyr394 is detected only in AD and other tauopathies. The nitration of these Tyr residues alters the conformation of tau, reducing its binding to microtubules and, depending on the nitration sites, can either promote or inhibit aggregation (Reyes et al., 2012).

Notably, tau is known to be ubiquitinated through Lys48 linkages by CHIP or tumor necrosis factor receptor-associated factor 6 (TRAF6) for proteasomal degradation (Babu et al., 2005; Petrucelli et al., 2004; Shimura et al., 2004). Thus, truncated tau missing the N-termini, including the Lys48 may bypass the degradation machinery process normal tau, and even undermine the degrading machinery of tau.

Finally, *in vitro* studies have shown that tau is also a substrate of sumoylation, with Lys340 being the major sumoylation site (Dorval and Fraser, 2006). Recent research has shown that that sumoylation of tau by small ubiquitin-like modifier protein 1 (SUMO-1) counteract against ubiquitination, and is correlates with tau hyperphosphorylation in HEK cells. Moreover, in AD brains, SUMO-1 colocalized with phosphorylated tau. In other words, tau sumoylation promotes its phosphorylation and inhibits the ubiquitination-mediated tau degradation (Luo et al., 2014).

1.5 Axonal transport impairment in tauopathies

Axonal and cell body accumulations of organelles and other proteins frequently occurs in neurodegenerative disease, leading to the appearance of axonal swellings and spheroids (Millecamps and Julien, 2013). Such pathologies suggest that defective functioning of axonal transport may contribute to disease. Axonal transport requires intact rails (microtubules), functional engines (kinesin and dynein), proper

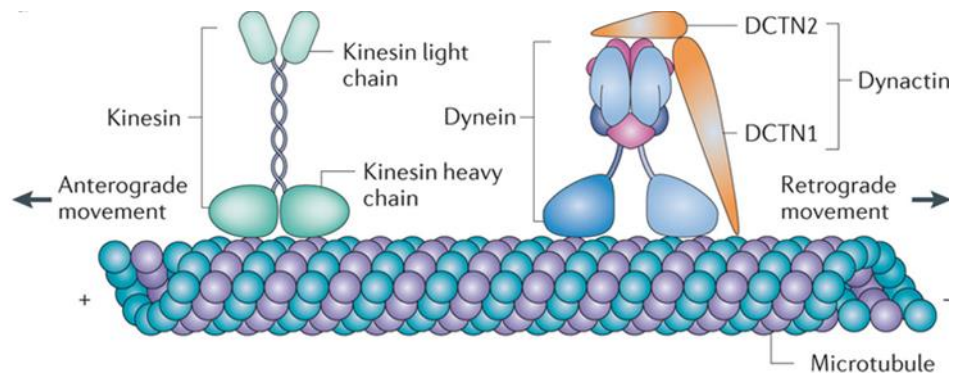
cargo loading (for example, to inhibit their attachment to motors), and sufficient ATP fuel supplied by mitochondria. Thus, each of these four components of the axonal transport system can be a target of pathogenic proteins (De Vos et al., 2008). Indeed, axonal transport defects have now been described as an early pathological feature in a variety of animal models of AD and tauopathies (Ballatore et al., 2007). Neurons containing PHFs exhibit severely impaired anterograde transport along axons as well as in basal dendrites; transport in apical dendrites is also impaired but in a retrograde-specific manner (De Vos et al., 2008; Millecamps and Julien, 2013).

1.5.1 Microtubule-based axonal transport

The delivery of cellular components to their action sites relies on long-range microtubule-based axonal transport (Fig 1.9). Microtubules composed of α -tubulin and β -tubulin heterodimers continuously undergo polymerisation and depolymerisation at the centrosome. In neurons, microtubules are polarised in axons and stabilised by microtubule-associated proteins, including tau. With ATP as the source of energy, cargoes are moved along the microtubule network by molecular motors. Molecular motors can be divided in to two groups, with transport toward the synapse (anterograde) mediated by kinesins and transport of most cargoes toward the cell body (retrograde) mediated by cytoplasmic dynein (Millecamps and Julien, 2013). *In vivo* studies revealed that the axonal transport rate of cytoskeleton components depends on the density of the stationary cytoskeletal network in axons (Millecamps and Julien, 2013).

Based on the bulk velocity of cargo movement, axonal transport is catalogued into fast and slow axonal transport. Vesicular cargoes such as vesicles, lysosomes and mitochondria move by fast axonal transport, while cytoskeleton components such as neurofilaments move in slow axonal transport (Lasek et al., 1984).

Figure 1.9 Components of the microtubule-based axonal transport system



Kinesin and dynein complexes govern the axonal transport of most cargoes. Kinesin is a heterotetramer composed of two heavy chains and two light chains, moves cargoes in the anterograde direction along axons (towards the axon tip). In contrast, dynein complexes, which comprise the dynein heavy, intermediate, intermediate light and light chains move cargoes in the retrograde direction (that is, towards the minus end of the microtubules). Dynactin is a complex composed of several dynactin subunits, such as dynactin subunit 1 and 2 (DCTN1 and DCTN2), in charge of the attachment of the cargo onto dynein (Millecamps and Julien, 2013).

It has also been suggested that dynein- and kinesin-mediated transport is modulated by local gradients of microtubule-associated proteins such as tau, in axons or by microtubule post-translational modification. Tubulin acetylation at Lys40 has been shown to enhance the recruitment of kinesin 1 to microtubules and promote anterograde transport of JNK-interacting protein 1 (JIP1) in neuronal cells (Reed et al., 2006). On the other hand, *in vitro* approaches suggest that a low concentration of tau in the proximal segment of the axon facilitates kinesin-mediated transport, whereas a high concentration of tau in the axon terminal is favourable for dynein-mediated transport (Shea et al., 2004). Tau promotes the detachment of kinesin from microtubules and restricts the distance travelled by kinesin along microtubules rich in GTP-tubulin, whereas it can enhance kinesin velocity along microtubules composed of GDP-tubulin (Dixit et al., 2008; Vershinin et al., 2007). The absence of *in vivo* evidence supporting these findings suggests more complicated regulatory machinery wherein multiple microtubule associated proteins are involved apart from tau (Vershinin et al., 2007). Adding another layer of complexity, tau may also

modulate axonal transport indirectly through altering tubulin acetylation (Perdiz et al., 2011).

1.5.2 Tau-induced deficits in axonal transport

Recent work supports the idea that tau may affect axonal transport through two highly inter-related ways: compromising the structure of microtubules and disrupting axonal transport machinery.

The collapse of microtubules is an important event during neurofibrillary degeneration induced by the aggregation of tau proteins in neurons. Abnormal modification of tau, such as increased phosphorylation, truncation and acetylation, impairs the interaction of tau with pre-existing microtubules and reduces the ability of tau to stabilise microtubules. Moreover, a reduced ability of pathological tau to promote microtubule assembly and form organised arrays was also found (Goedert and Jakes, 2005). Further, overexpression of mutant or wild-type tau in mice results in dendritic mis-sorting of tau and destabilisation of microtubules, an effect that was rescued by microtubule-stabilising drugs (Zhang et al., 2012a). Loss of tau function therefore, leads to a loss of the microtubule rails that are required for efficient axonal transport. In addition, reduced tubulin acetylation has been observed in neurons containing NFTs in AD brain, proposing tubulin acetylation as a new player in impairment of axonal transport.

Tau can interfere with the binding of motor proteins, both dynein and kinesin to microtubules, reducing the binding frequency as well as the mobility of these two proteins, which slows both anterograde and retrograde transport (Seitz et al., 2002). Overexpression and mis-localisation of tau appear to modulate kinesin 1 based transport by direct inhibition of motors on microtubule tracts (Ebner et al., 1998). Moreover, *in vitro* investigations have revealed that tau inhibits kinesin-mediated transport, not only by reducing the travel distance of single-kinesin, but also leads to a modest reduction in the single-kinesin velocity. (Dixit et al., 2008; Ebner et al., 1998; Stamer et al., 2002). Tau can reduce the number of motors that are engaged with cargoes and thereby interfere with axonal transport of cargoes (Vershinin et al., 2007). Protein levels of both the kinesin motor-mediated axonal transport machinery and of the dynein-mediated retrograde transport machinery are reduced in AD (Morel et al., 2012). This reduction, especially decreased level of kinesin light chain and dynein intermediate chain will compromise the capacity of the motors. Tau can also sequester available kinesin and thereby limit axonal transport of other cargoes

(Konzack et al., 2007; Utton et al., 2005). Tau can regulate the release of cargo vesicles from kinesin chains by activating protein phosphatase 1 (PP1) and GSK3 β (Kanaan et al., 2011). It is also found that amino acids 2-18 of tau are necessary and sufficient for the activation of this pathway. Thus, increased activation of GSK3 β can also contribute to the deficit by aberrant phosphorylation of light chain of kinesin, resulting in premature release of kinesin from its cargoes. It was further found that tau is able to trap kinesin adapter-molecule JIP1 from microtubules to the neuronal soma (Ittner et al., 2008; Ittner et al., 2009).

Consequently, pathological tau can not only compromise the structural basis of synapses, but also inhibit transport of other cargoes to the synapse, eventually causing synaptic degeneration. Meanwhile, displaced and accumulating organelles, such as mitochondria, may results in neuronal energy deprivation and oxidative stress, fuelling the progression of pathology and neuronal demise in AD and related disorders.

1.6 The UPR and the tauopathies

The unfolded protein response (UPR) is a stress response of the endoplasmic reticulum (ER) to internal and external insults, especially protein misfolding. Over the last decade, increasing studies on neurodegeneration have suggested the involvement of the UPR in human neuropathology in neurological disease, as well as in cell and mouse models of neurodegeneration.

1.6.1 Endoplasmic reticulum stress and the UPR

The endoplasmic reticulum (ER) is an important organelle wherein nascent proteins are folded with the assistance of ER chaperones (i.e. molecular chaperones and folding enzymes). On the other hand, unfolded and misfolded proteins in the ER are translocated to the 26S proteasome, where they are degraded by ER-associated degradation (ERAD) (Nakatsukasa and Brodsky, 2008). The ER is also a major intracellular calcium storage compartment and it is a critical player in the maintenance of cellular calcium homeostasis. The ER also participates in lipid biosynthesis, including lipid membrane synthesis, and controlling the synthesis of cholesterol and other membrane lipid components (Hetz and Mollereau, 2014).

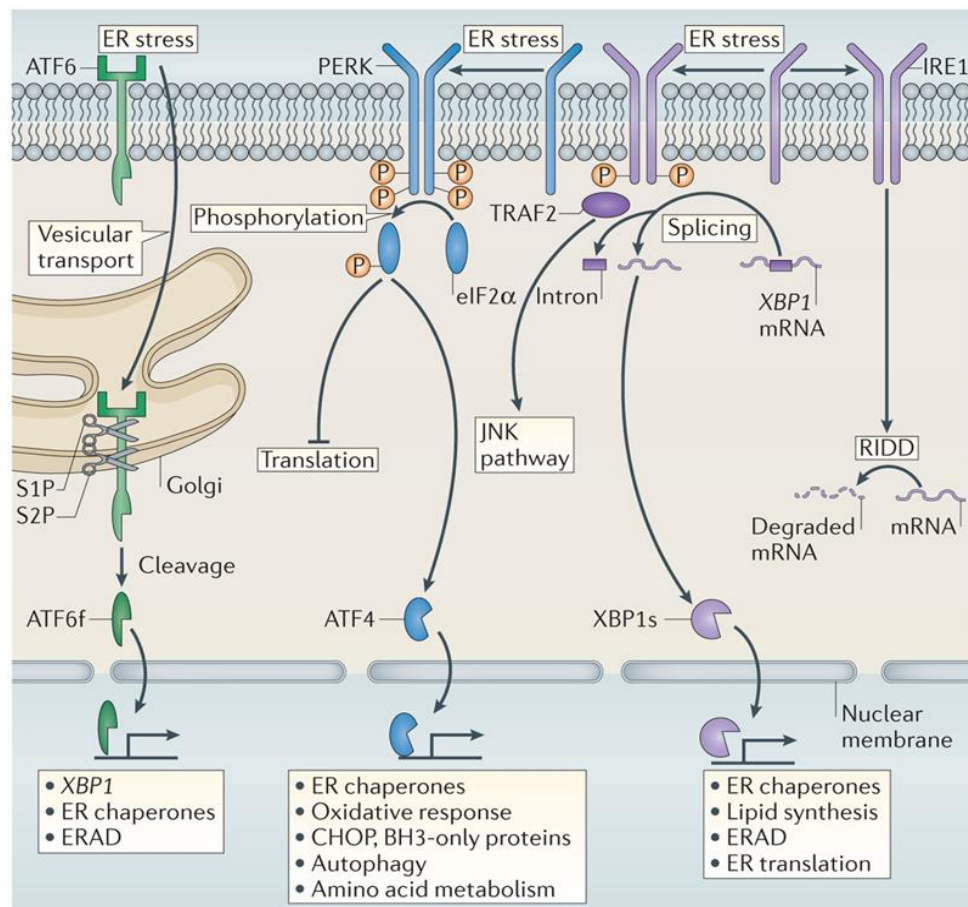
However, when the normal function of the ER is perturbed, either by external or internal insults, namely ER stress, a stress response of ER, the UPR will be

activated. ER stress includes disturbances in cellular redox regulation caused by hypoxia, glucose deprivation, disruption of Ca^{2+} metabolism causing impaired functions of Ca^{2+} dependent chaperones, viral infections which overload the ER with virus encoded proteins, and protein mutations that hamper adequate folding (Schonthal, 2012).

Initiation of the UPR results in signalling through three branches, each of which utilises one of the three ER stress sensors inositol-requiring transmembrane kinase/endonuclease 1 (IRE1), activating transcription factor 6 (ATF6) and (PKR)-like endoplasmic reticulum kinase (PERK), triggering a signalling cascade, in an attempt to re-establish homeostasis and normal ER function (Fig.1.10). IRE1 is a transmembrane kinase/endonuclease (RNase). Activation of IRE1 initiates the splicing of X-box binding protein 1 (XBP1) mRNA. A 26-nucleotide intron of XBP1 mRNA is spliced out under ER stress conditions, leading to a frame shift and expression of spliced X-box-binding protein 1 (sXBP1), which drives transcription of genes including ER chaperones, which facilitating correct protein folding in the ER (Ron and Hubbard, 2008; Shaffer et al., 2004). PERK is a transmembrane protein kinase that phosphorylates and activates eukaryotic initiation factor 2 α (eIF2 α). Activated eIF2 α blocks the loading of mRNA to ribosomes during the initiation of transcription, leading to reduced protein synthesis, to counteract ER protein overload (Fels and Koumenis, 2006; Smith and Mallucci, 2016). ATF6 is an ER-resident transmembrane protein. Upon activation, the cytoplasmic domain of ATF6 is released from the ER, it is then cleaved into a 50 kDa fragment in Golgi and translocated to the nucleus. Increased nuclear ATF6 enhances expression of genes encoding proteins that augment ER protein folding capacity (Sommer and Jarosch, 2002). Following initiation of the UPR, the outcome depends on the level of ER stress. Mild ER stress will result in an adaptive ER response during which cells recover to their normal physiological state. However, prolonged and severe ER stress results in cell death, which is discussed below (Hetz and Mollereau, 2014; Oakes and Papa, 2015).

Although UPR activation was initially regarded as a protective cellular mechanism, evidence has shown that prolonged activation of the UPR is deleterious to cells, inducing apoptosis and insulin resistance (Srinivasan et al., 2005; van der Kallen et al., 2009). Furthermore, links between ER stress/UPR and A β and tau pathology have also been drawn, and the evidence is emerging to indicate that ER stress may be a contributor to neurodegeneration (Scheper and Hoozemans, 2015).

Figure 1.10 Overviews of the three signalling branches of the UPR



Upon activation of the UPR, ATF6 is transported to the Golgi, where it is cleaved by site 1 protease (S1P) and site 2 protease (S2P), releasing the cytosolic ATF6 fragment (ATF6-p50), which operates as a transcription factor. ATF6-p50 induces genes required for ER associated degradation (ERAD) and XBP1 mRNA levels. In the PERK branch of the UPR, activated PERK phosphorylates eIF2α, attenuating protein translation with the exception of UPR components such as ATF4. ATF4 is a transcription factor governing the expression of ER chaperones, genes related to autophagy, redox control and amino acid metabolism as well as genes related to apoptosis, including CCAAT-enhancer-binding proteins homologous protein (CHOP). Active IRE1 induces splicing of XBP1 mRNA, producing sXBP1, which upregulates the transcription of ER chaperones, components of the ERAD pathway and regulators of lipid synthesis. IRE1 is also associated with tumor necrosis factor receptor-associated factor 2 (TRAF2) and induces c-Jun N-terminal kinases (JNK) activation, thereby modulating autophagy and apoptosis independent of XBP1 (Hetz and Mollereau, 2014).

1.6.2 Crosstalk between ER stress/UPR and amyloid- β

Emerging evidence indicates that the interplay between ER stress and disease-associated proteins plays a key role driving the progression of several neurodegenerative diseases. Markers of ER stress-induced UPR activation have been found in post-mortem brain from affected patients and in animal models of AD, PD, ALS, HD, and transmissible spongiform encephalopathies (Hetz and Mollereau, 2014; Hoozemans et al., 2012).

A β is able to trigger an ER stress response leading to synaptic and neuronal loss (Costa et al., 2010; Fonseca et al., 2013). *In vitro* studies have demonstrated that A β exposure increases the activation of ER stress response markers (Chafekar et al., 2008). Data from both *in vitro* studies and neurons has shown that caspase 4, an ER stress-specific caspase, may be involved in AD pathogenesis (Hitomi et al., 2004). Furthermore, in cultured cortical neurons, A β stabilises and activates caspase 12 by inhibiting proteasome activity, thus increasing the A β -induced cell death that is triggered by the ER stress-activated caspase 12 (Nakagawa et al., 2000). Supporting this notion, in cortical and hippocampal synaptosomes isolated from 3 \times Tg-AD mice, A β induces synaptic toxicity in a caspase 12-dependent manner (Quiroz-Baez et al., 2011). ER stress has also been implicated as a mediator of A β toxicity by promoting mitochondrial and ER dysfunction (Ferreiro et al., 2012). Presenilin 1, one building block of γ -secretase complex was shown to be upregulated by an ATF4-dependent process under ER stress conditions, increasing γ -secretase activity (Ohta et al., 2011). Given the role of γ -secretase as the generator of A β peptides, these results implicate ER stress/UPR as a driver of A β pathology and downstream caspase activation that results in cell death.

5.1.3 Crosstalk between ER stress/UPR and tau

Associations between ER stress and tau pathology have been described in several tau-related neurological disorders, including AD, non-AD tauopathies, PD, prion diseases, ALS and HD (Table 1.3) (Ferreiro and Pereira, 2012; Hoozemans and Scheper, 2012). In AD brain, increases in UPR markers closely correlate with the presence of phosphorylated tau and GSK3 β (Nijholt et al., 2012). This suggests that prolonged UPR activation in neurons is involved in tau phosphorylation and indicates the emergence of the UPR as an early stage event during AD pathogenesis (Hoozemans et al., 2009). UPR activation has also been implicated in sporadic tauopathies such as PSP, PiD and FTLD-tau (Nijholt et al., 2012). Furthermore, it has been suggested that there may be a vicious cycle wherein UPR

activation contributes to tau phosphorylation and that increased tau phosphorylation also activates the UPR (Ho et al., 2012). Increased active PERK and eIF2 α , splicing of XBP1 mRNA, and elevated CHOP mRNA have been found in primary neurons treated with the protein phosphatase inhibitor, okadaic acid, which also increases tau phosphorylation (Ho et al., 2012). In turn, thapsigargin, a pharmacological UPR inducer, stimulates tau phosphorylation at Thr231, Ser262, and Ser396 (Ho et al., 2012).

Table 1.3 The UPR in neurodegenerative diseases

Neurodegenerative disease	UPR marker	Technique, brain area	Association with pathology	References
Alzheimer's disease	GRP78	IHC, hippocampus	Increased in AD, associated with healthy neurons	(Hamos et al., 1991)
	p-eIF2 α	IHC, hippocampus, entorhinal cortex	Increased in AD, associated with GVD	(Chang et al., 2002)
	pPERK, GRP78	IHC and WB, hippocampus and temporal cortex	Increased in AD	(Hoozemans et al., 2005)
	pPERK, p-eIF2 α	IHC, hippocampus, frontal cortex	Increased in AD, associated with abnormally phosphorylated tau	(Unterberger et al., 2006)
	hHRD1	IHC, hippocampus	Increased in AD	(Hou et al., 2006)

	pPERK, pIRE1, p-eIF2 α	IHC, hippocampus	Increased in AD, associated with GVD and abnormally phosphorylated tau	(Hoozemans et al., 2009)
	pPERK, p-eIF2 α	IHC, pons medulla, hippocampus	Increased in affected brain areas	(Stutzbach et al., 2013)
Tauopathy				
CBD/PSP	pPERK, p-eIF2 α	IHC, hippocampus, frontal cortex	Increased in CBD/PSP, associated with abnormally phosphorylated tau	(Unterberger et al., 2006)
FTLD-tau, PiD, PSP	pPERK, pIRE1	IHC, hippocampus, frontal cortex, temporal cortex	Increased in affected brain areas, associated with GVD and early tau pathology	(Nijholt et al., 2012)
PSP	pPERK, p- eIF2 α	IHC, pons medulla, hippocampus	Increased in affected brain areas	(Stutzbach et al., 2013)
Synucleinopathy				
Parkinson's disease	pPERK, p-eIF2 α	IHC, substantia nigra	Increased in PD, association with α - synuclein	(Hoozemans et al., 2007)

Multiple system atrophy	pPERK, pIRE1, p-eIF2 α	IHC, middle cerebellar peduncle, white matter of cerebellum, pontocerebellar fibres, striatum, GCI	Association with α -synuclein inclusions, abnormally phosphorylated tau, pTDP-43 and GVD	(Makioka et al., 2010)
Prion disease (sCJD, vCJD)	GRP58, GRP78, GRP94	WB, cortex	Increased levels in CJD	(Hetz et al., 2003)
Amyotrophic lateral sclerosis	pPERK, p-eIF2 α	IHC, hippocampus, frontal cortex	No increase in CJD	(Unterberger et al., 2006)
	PERK, ATF6, IRE1, GRP78, Erp57, PDI, CHOP, caspase 4	WB, spinal cord	PERK, ATF6, IRE1 and caspase 4 are increased in ALS. GRP78, Erp57, PDI and CHOP are unchanged	(Atkin et al., 2008)
	CHOP	IHC, spinal cord	CHOP is increased in ALS	(Ito et al., 2009)
	GRP78	IHC, spinal cord	GRP78 is increased in ALS	(Sasaki, 2010)
	p-eIF2 α	IHC, WB, spinal cord	p-eIF2 α is increased in ALS	(Ilieva et al., 2007)
	sXBP1, ATF4, GRP58	WB, spinal cord	sXBP1, ATF4 and GRP58 are increased in ALS	(Hetz et al., 2009)

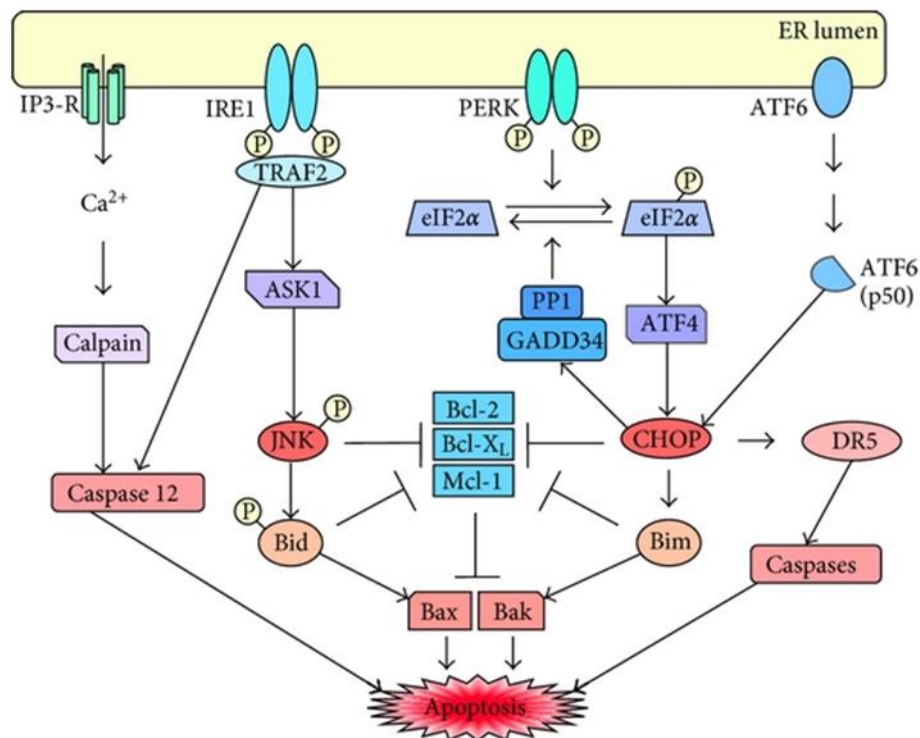
Repeat expansion diseases				
Huntington's disease	GRP78, CHOP	PCR, parietal cortex	Increased expression in HD	(Carnemolla et al., 2009)
	ATF6 α	IHC, WB, caudate putamen	Impaired ATF6 α processing	(Fernandez-Fernandez et al., 2011)
	pIRE1, GRP78	WB, striatum	Increased in HD	(Lee et al., 2012)
	sXBP1, ATF4, CHOP, GRP78	WB, striatum	Increased sXBP1, no changes in ATF4, CHOP, GRP78	(Vidal et al., 2012)
C9ALS	ATF4, CHOP, GRP78	PCR, frontal cortex	Increased ATF4 and CHOP in C9ALS, no change in GRP78	(Zhang et al., 2014a)

UPR: unfolded protein response; GRP: glucose-regulated protein; IHC: immunohistochemistry; AD: Alzheimer's disease, p-eIF2 α : phosphorylated eukaryotic initiation factor 2 alpha; GVD: granulovacuolar degeneration; pPERK: phosphorylated protein kinase R (PKR)-like endoplasmic reticulum kinase; WB: Western blot analysis; hHRD1: Human 3-hydroxy-3-methylglutaryl-coenzyme A reductase degradation 1; pIRE1: phosphorylated inositol requiring enzyme 1; CBD: corticobasal degeneration; PSP: progressive supranuclear palsy; Neuropathology of frontotemporal lobar degeneration-tau; GCI: gyrus cingula; s/vCJD: sporadic/variant Creutzfeldt–Jakob disease; ATF: activating transcription factor; PDI: protein disulfide isomerase; CHOP: C/EBP homologous protein; sXBP1: spliced X-box binding protein 1; ALS: amyotrophic lateral sclerosis; PCR: polymerase chain reaction; HD: Huntington's disease; C9ALS: ALS with the open reading frame 72 on chromosome 9 repeat expansion; GRP78: 78 kDa glucose-regulated protein (Scheper and Hoozemans, 2015).

1.6.3 The UPR and apoptosis

In the context of neurodegenerative disease, neuronal cell loss is a hallmark of the progression of pathology. Similarly, cell death is implicated in a wide variety of diseases including ischemic injury (stroke, myocardial infarction), heart failure, and bipolar disorder (Kim et al., 2008). Recent data has indicated that ER stress triggers pro-apoptotic signals via several different mechanisms. Notably, activation of ER stress sensors initiates both the protective mechanism through activation of sXBP1 and the pro-apoptotic mechanism by activating CHOP and JNK (Lindholm et al., 2006).

Figure 1.11 Cell death signalling by the ER stress response/UPR



In sustained ER stress, a number of pro-apoptotic events begin to dominate and lead to apoptosis. Transcription factors ATF4 and ATF6-p50 fragment stimulate CHOP expression. CHOP inhibits anti-apoptotic proteins of the Bcl-2 family and stimulates pro-apoptotic Bim, leading to activation of pro-apoptotic Bax and Bak. CHOP also stimulates expression of cell surface death receptor 5 (DR5), which sensitises cells to pro-apoptotic stimuli, presumably via calibrating the extrinsic apoptotic pathway involving caspase-12. Similarly, activated JNK complements the pro-apoptotic efforts of CHOP. JNK becomes phosphorylated and activated by protein kinase ASK1 upon association of TRAF2 with activated IRE1.

Association of TRAF2 with activated IRE1 also leads to activation of caspase-12. Calcium release from the ER via IP3 receptors can activate calpains, which further stimulate caspase-12 activation via proteolytic cleavage of its inactive procaspase precursor (Schonthal, 2012).

Under pathological conditions, sustained accumulation of these pro-apoptotic factors overrides the protective effects of sXBP1, ultimately driving the cell death pathway (Fig 1.11). Recent findings have determined that all three branches of the UPR to be related to ER stress-induced apoptosis: (1) the pro-apoptotic pathway of CHOP transcription factor which is mainly induced via PERK/eIF2 α . (2) IRE1-mediated activation of apoptosis signal-regulating kinase 1(ASK1)/JNK, and (3) activation of caspase-12 (Kim et al., 2006). Both CHOP and JNK modulate the balance between pro- and anti-apoptotic members of the B-cell lymphoma 2 family (Bcl-2 family), enhancing the expression of pro-apoptotic Bak and Bax, resulting in permeabilisation of the outer mitochondrial membrane and execution of the intrinsic apoptotic process (Dhanasekaran and Reddy, 2008; Jager et al., 2012). Caspase-12 is activated by m-calpain in the cytoplasm, which is a consequence of calcium efflux from the ER following stress. These three pathways all culminate in a cascade of caspase activation and elicit the execution of ER stress-induced cell death (van der Kallen et al., 2009).

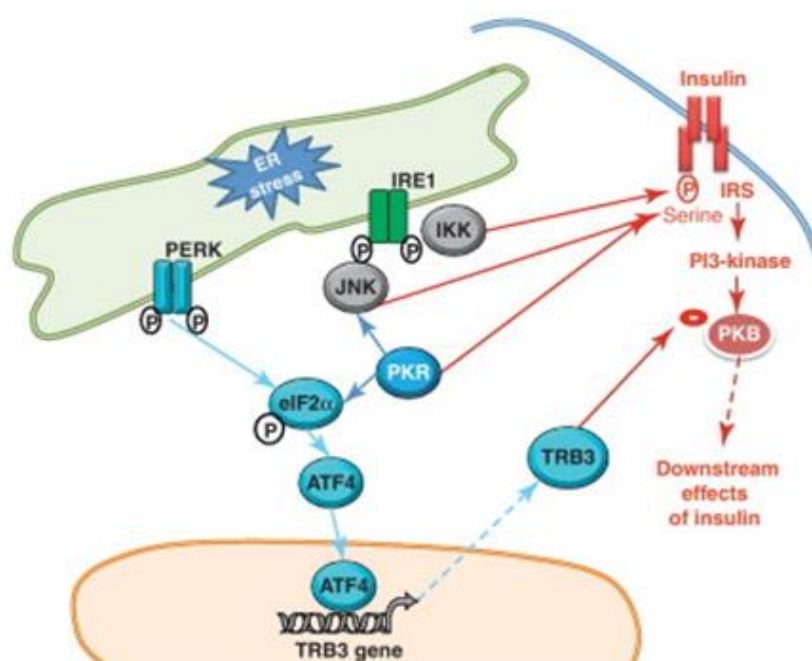
1.6.4 ER stress/UPR and insulin resistance

As shown in Fig 1.12, insulin binds to the insulin receptor and triggers its intrinsic tyrosine kinase activity. This kinase activity phosphorylates on tyrosine residues members of a conserved family of adaptor proteins termed as insulin receptor substrates 1-4 (IRS1-4). Phosphorylated IRS act as scaffolds that couple insulin receptor stimulation to downstream effectors, such as PI3K, enabling metabolic and transcriptional reprogramming of cells. Once IRS1 undergoes subsequent serine phosphorylation by JNK, IRS1 then dissociates from the insulin receptor, leading to decreased tyrosine phosphorylation. The interaction of IRS1 with PI3K is thus reduced, resulting in cellular insulin resistance (Gual et al., 2005).

Insulin resistance is a key pathological feature shared both by certain neurodegenerative diseases and by diabetes (van der Kallen et al., 2009). In the case of AD, it is becoming evident that A β oligomers disrupt normal brain insulin signalling, either directly, through disturbing the binding of insulin to its receptor, or indirectly, through pro-inflammatory mechanisms (Najem et al., 2014). In neurons,

exposure of A β oligomers triggers insulin receptor internalisation, reducing their number on the cell surface and resulting in the appearance of insulin resistance markers, such as downregulation of plasma membrane insulin receptors (IRs) or substantial loss of neuronal surface IR (De Felice et al., 2009; Zhao et al., 2008). Additionally, A β oligomers instigate aberrant activation of the tumor necrosis factor receptor-associated factor 2 (TRAF2)/JNK pathway, and IRS1 inhibition, in primary hippocampal neurons (Bomfim et al., 2012; Ma et al., 2009). IRS1 inhibition was also demonstrated in the brains of AD patients (Bomfim et al., 2012). Elevated pSer-IRS1 and activated JNK co-exist in post-mortem AD brain, which provides good evidence for establishing a relationship between A β oligomers, JNK activation and IRS1 inhibition (Talbot et al., 2012). Among the components in the cascade of insulin resistance, I κ B kinase (IKK) and JNK are also key players of inflammation and ER stress (Zhang and Kaufman, 2008). Thus, it is possible that the complex pathology of AD is formed of intertwined processes including insulin resistance, A β /tau pathologies and neuroinflammation.

Figure 1.12 The UPR can directly promote insulin resistance



Upon initiation of the UPR, IRE1 activation leads to the recruitment of JNK and IKK, both of which impair insulin signalling by phosphorylating IRS1 on serine residues. ER stress exposure also can inhibit insulin signalling directly by phosphorylating IRS1 on serine residues, or indirectly through JNK. Finally, tribbles-related protein 3 (TRB3), an ER stress activated protein via the PERK branch of the UPR, also impairs insulin signalling by inhibiting Akt (Flamment et al., 2012).

1.6.5 Measuring UPR activation in cells

The large number of cellular factors involved in the process of UPR activation provides a number of different measures by which to monitor the progression of the UPR in cells (Osłowski and Urano, 2011). Upon ER stress, IRE1 and PERK, two of the stress master regulators of the UPR, undergo autophosphorylation. Thus, measuring the phosphorylation of IRE1 and PERK is a good means by which to measure the activation state of the UPR. As the downstream substrate of PERK, measuring eIF2 α phosphorylation also provides information on the activation of the PERK branch of the UPR. However, PERK is not the only eIF2 α kinase (Donnelly et al., 2013). For ATF6, its activation can be assessed through translocation of the GFP-ATF6 fusion protein by microscopy from ER to nucleus or by detection of the cleaved 50 kDa fragment of ATF6 on western blots. Downstream of the three master regulators, XBP1 splicing can be examined at the RNA level using quantitative real-time PCR as well as at the protein level on western blots.

Finally, as the ultimate outcome of UPR, monitoring cell death/apoptosis can also be helpful for getting a comprehensive understanding of UPR, especially the mechanisms underlying the pathogenesis of ER stress-related disorders. There is a diverse array of components in the UPR cascade that serve as markers of UPR mediated apoptosis. ASK1 and JNK are serine/threonine protein kinases and therefore, their activation levels can be measured using phospho-specific antibodies. CHOP is a pro-apoptotic transcription factor of the UPR and because its baseline expression is low, CHOP upregulation and activation can be measured on western blots, or using real-time PCR. Pro-apoptotic Bax and Bak can also be measured by immunoblot. Moreover, standard apoptosis markers, including cleaved caspase-3, DNA fragmentation and cell viability assays can also be used for investigating UPR-mediated cell death.

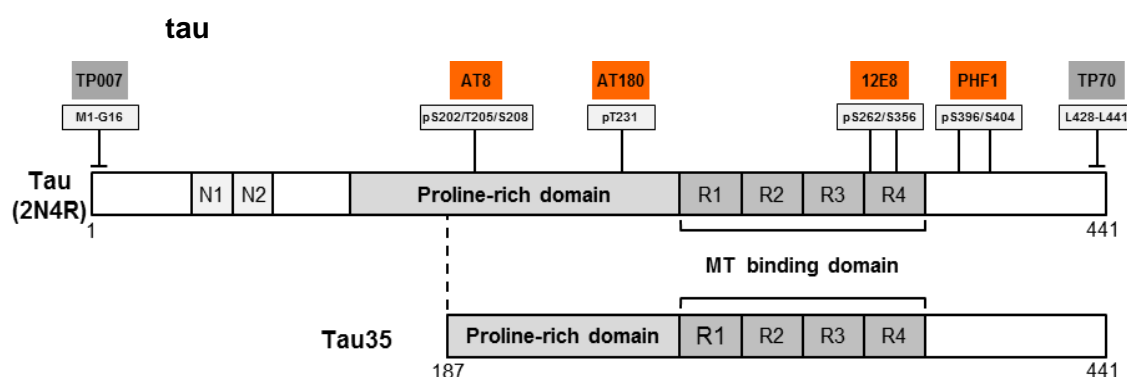
In summary, ER stress and activation of the UPR play central roles, linking misfolded proteins with the pathological changes observed in neurodegenerative disease. Although the link between A β and onset of ER stress has been intensively studied, the role of tau in driving UPR activation is not yet well understood. Thus, investigating the UPR in models of tauopathy would be expected to shed new light on the critical mechanisms underlying disease pathogenesis.

1.7 Summary

Although enormous progress has been made towards the full elucidation of the pathogenesis of tauopathies, there are still many open questions regarding the molecular mechanisms underlying the onset and development of such diseases. This is especially true for the changes that lead from normal functional tau to pathogenic forms of aggregated tau in sporadic tauopathies that are independent of A β . Recent reports have proposed an important role for tau truncation in promoting pathological changes in tau. The roles of key protein kinases, such as GSK3 β , have also been highlighted, since they may be involved in tau-mediated neurodegeneration through several different routes, from perturbing normal function tau, facilitating tau misfolding, disrupting axonal transport to finally mediating cell death.

From a systemic perspective, the activation status of the UPR is also critical for the maintenance of the physiological equilibrium, disruption to which might induce a pathological outcome. Given malfunctioning tau as a primary cause of tauopathy, a properly regulated UPR may be able to minimise damage to neurons. On the contrary, prolonged, over-activation of the UPR will aberrantly interfere with multiple pathways and aggravate degeneration. Thus, detailed investigation of how truncated tau can trigger abnormal processes in cells, as well understanding the interplay between tau, protein kinase activation and the UPR, will provide valuable information to enhance knowledge of the molecular mechanisms involved in the progression of tauopathies.

Figure 1.13 Schematic representation of Tau35 in comparison to 2N4R human



2N4R tau (amino acids 1-441) contains two inserts (N1, N2), followed by proline-rich domain and the MT binding domain, consist of four MTB repeats (R1-R4). Tau35 (amino acids 187-441) retains majority of the proline-rich domain, four MTB repeats and an intact C-terminus. The epitopes of the phospho-dependent (orange boxes) and region-specific (grey boxes) tau antibodies used in this study are indicated above 2N4R tau (Bondulich et al., 2016).

1.8 Aims of this thesis

In 2008, initial data from Wray and colleagues described a 35 kDa C-terminal tau fragment (Tau35), lacking the N-terminus of tau but containing four microtubule-binding repeats (4R) (Fig 1.13). Tau35 is readily detectable in PSP, CBD and FTLD-tau brain but is absent from health control brain. Subsequent findings in a transgenic mouse model expressing Tau35 showed that this model manifested key features of tauopathies, particularly accumulation of abnormally phosphorylated tau along with dysregulated GSK3 β , progressive cognitive and motor deficits, and loss of synaptic protein (Bondulich et al., 2016). This study investigates the molecular mechanisms that result in these phenotypes as well as exploring new aspects of the pathogenesis underlying Tau35-mediated dysfunction. The specific objectives are:

- To establish a CHO cell model stably expressing a disease-associated tau fragment (Tau35), present in human tauopathies.
- To assess the biochemical properties of Tau35 in cells and to evaluate the impact of tau truncation on the normal function of tau.
- To determine whether the deleterious effects of Tau35 identified in mice are related to changes in the activation state of the UPR.

Chapter 2 Materials and methods

2.1 Materials

All reagents and chemicals were obtained from Sigma-Aldrich unless otherwise indicated.

Plasmids

Plasmids encoding full-length human tau (FL-tau) or Tau35 were generated in pcDNA 3.1D/V5-His-TOPO vector (Invitrogen), which carries a neomycin resistance gene, a V5 epitope, a 6× His tag and the promoter from cytomegalovirus as shown in Fig. 2.1. cDNA sequences corresponding to hTau40 (Derkinderen et al., 2005) and tau35 were each inserted into the multiple cloning site of this vector at BamHI – XbaI. Alpha-tubulin N-acetyltransferase (ATA-1)/MEC-17 plasmid was a kind gift from Dr Kurt De Vos (University of Sheffield). The cDNA sequence of the murine MEC-17 orthologue was inserted into pCMV-SPORT6 plasmid (Godena et al., 2014).

Figure 2.1 Plasmid details for constructs encoding FL-tau/Tau35

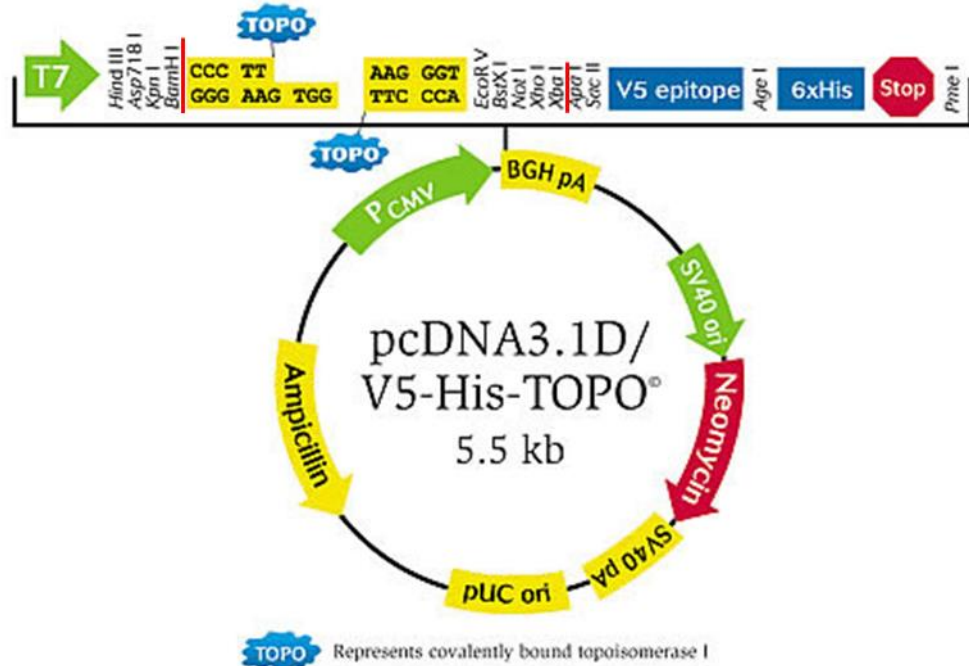


Fig 2.1. cDNA sequences corresponding to full-length human tau (FL-tau) (Derkinderen et al., 2005) and Tau35 were each inserted into the multiple cloning site of pcDNA 3.1D/V5-His-TOPO at BamHI – XbaI. The translated products are either full-length human tau (amino acids 1-441) or amino acids 187-441 of full-length tau (Tau35), each with an additional N-terminal methionine and C-terminal V5/6xHis tag. The plasmid map is from Invitrogen.

General buffers

Phosphate-buffered saline (PBS)	1 PBS tablet dissolved in 200ml ultra-high purity (UHP) water to give a final concentration of 10 mM Na_2HPO_4 , pH 7.4 2 mM KH_2PO_4 137 mM NaCl 2.7 mM KCl
Tris-buffered saline (TBS)	25 mM Tris-HCl, pH 7.4 150 mM NaCl 2 mM KCl
2× Laemmli sample buffer	125 mM Tris-HCl, pH 6.8 4% (w/v) Sodium dodecyl sulfate(SDS) 100 mM Dithiothreitol 20% (v/v) Glycerol 0.01% (w/v) Bromophenol blue
Protease inhibitor cocktail (7×)	1× protease inhibitor cocktail tablets (Roche) 1.5 ml H_2O dilute to 1× before use
Phosphatase inhibitor cocktail (1×)	20 mM NaF 1 mM Na_3VO_4 1 mM $\text{Na}_2\text{P}_2\text{O}_4$ added freshly before use

Cell culture

Ham's F-12 (PAA Laboratories Ltd)	F-12 Nutrient Medium
CHO cell culture medium	Ham's F-12 medium (PAA Laboratories Ltd) 10% (v/v) Foetal bovine serum (FBS) 2 mM L-Glutamine (PAA Laboratories Ltd) 100 U/ml Penicillin (PAA Laboratories Ltd) 100 µg/ml Streptomycin (PAA Laboratories Ltd)
Trypan Blue	0.4% (w/v) Trypan blue solution
HEPES buffer	10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4
HBSS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (Invitrogen)	Hank's Balanced Salt Solution without $\text{Ca}^{2+}/\text{Mg}^{2+}$

HBSS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (Invitrogen)	Hank's Balanced Salt Solution with $\text{Ca}^{2+}/\text{Mg}^{2+}$
G418 stock solution	40 mg/ml G418 (Santa Cruz Biotechnology) in 0.1 M HEPES buffer, pH 7.4 Sterilised by 0.2µm filtration and stored at -20 °C
G418 selection medium	Ham's F-12 medium containing 800 µg/ml G418
Cell freezing medium	10% (v/v) Dimethyl sulfoxide (DMSO) in Ham's F-12

Sarkosyl/Triton X-100 insoluble fractionation

H buffer (homogenisation buffer)	20 mM Mes, pH 6.8 800 mM NaCl 1 mM MgCl_2 2 mM EGTA 10 mM NaH_2PO_4 20 mM NaF The following was added at time of use: protease inhibitor cocktail
S buffer (fractionation buffer)	500 mM NaCl 10% Sucrose 1% (w/v) N-Lauroylsarcosine (Sarkosyl) or 1% (w/v) Triton X-100

***In situ* microtubule binding assay**

Microtubule stabilizing buffer	80 mM PIPES, pH 6.8 1 mM MgCl_2 1 mM EDTA 0.5% (v/v) Triton X-100 30% (v/v) Glycerol The following were added before use: 0.5 µM Okadaic Acid (Santa Cruz Biotechnology) 10 µM Taxol 1 mM GTP protease inhibitor cocktail
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Membrane associated protein extraction

Hypotonic buffer	10 mM NaHCO ₃ 20 µg/ml DNase I The following was added at time of use: protease inhibitor cocktail phosphatase inhibitor cocktail
Wash buffer	100 mM Na ₂ CO ₃ , pH 11.5

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (WB)

4x Resolving Buffer	1.5 M Tris-HCl, pH 8.8 0.4% (w/v) SDS
4x Stacking Buffer	0.5 M Tris-Cl, pH 6.8 0.4% (w/v) SDS
10% Resolving gel	10% (w/v) Acrylamide (Geneflow) 25% (v/v) Resolving Buffer 0.01% (w/v) Ammonium persulphate 0.1% (v/v) N,N,N',N',Tetramethylethylenediamine (Geneflow)
4% Stacking gel	4% (v/v) Stacking gel buffer 10% (w/v) Acrylamide (Geneflow) 0.01% (w/v) Ammonium persulphate (Geneflow) 0.1% (v/v) N,N,N',N',Tetramethylethylenediamine (Geneflow)
Running buffer	25 mM Tris 192 mM Glycine 0.1% (w/v) SDS
Molecular weight markers	Precision Plus Protein All Blue Standards (Bio-Rad) Molecular weight sizes (Tris-Glycine gel): 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa
Transfer buffer	25 mM Tris-HCl, pH 8.3 192 mM Glycine 20% (v/v) Methanol
PBS-T	PBS 0.2% (v/v) Tween-20

TBS-T	20 mM Tris-HCl, pH 7.4 150 mM NaCl 0.2% (v/v) Tween-20
Blocking buffer	TBS-T or PBS-T containing 5% (w/v) dried skimmed milk or 5% (w/v) bovine serum albumin
Odyssey blocking buffer	Li-Cor Biosciences

Immunocytochemistry (ICC)

4% PFA	16% (w/v) Paraformaldehyde (PFA) in PBS, pH 7.4
Blocking buffer (FBS)	10% (w/v) FBS 1% (v/v) Triton X-100 0.01% (w/v) NaN ₃ in PBS, pH 7.4
Blocking buffer (Milk)	3% (w/v) defatted milk 1% (v/v) Triton X-100 0.01% (w/v) NaN ₃ in PBS, pH 7.4
Permeabilisation buffer	0.25% (v/v) Triton-X100 in PBS
Antibody dilution buffer	10% (v/v) blocking buffer (FBS or milk) in PBS, pH 7.4
Hoechst solution	0.5% (v/v) Hoechst 33342 in PBS, pH 7.4

Live/Dead Assay

LIVE/DEAD[®] Fixable Far-red Dead Cell Stain Kit (Life Technologies, Paisley, UK).

Antibodies

Primary antibodies

Antibody	Epitope	Species	Dilution	Source
K9JA	C-terminal half of tau	Rabbit polyclonal	WB 1/10,000 ICC 1/1,000	DAKO
Anti-V5	V5 tag (GKPIPPELLGLDST)	Mouse monoclonal	WB 1/5,000 ICC 1/1,000	Invitrogen

TP007	Tau N-terminus residues 1-16	Rabbit polyclonal	WB 1/10,000 ICC 1/2,000	(Davis et al., 1995)
TP70	Tau C-terminus residues 427-441	Rabbit polyclonal	1/5,000	(Brion et al., 1993)
PHF1	Tau phosphorylated at Ser396/Ser404	Mouse monoclonal	2,000	Peter Davies (Albert Einstein College of Medicine)
12E8	Tau phosphorylated at Ser262/Ser356	Mouse monoclonal	1/10,000	Prothena Corporation
AT8	Tau phosphorylated at Ser202/Thr205/Ser208	Mouse monoclonal	1/3,000	Thermo Scientific
AT180	Tau phosphorylated at Thr231	Mouse monoclonal	1/500	Innogenetics
Anti-Actin (AC15)	Beta-actin	Mouse monoclonal	1/10,000	Abcam
Anti-Actin (Ab8227)	Beta-actin	Rabbit polyclonal	1/10,000	Abcam
DM1A	Alpha-tubulin	Mouse monoclonal	1/1,000	Sigma
Acetylated tubulin	Tubulin acetylated at Lys40	Mouse monoclonal	1/2,000	Santa Cruz Biotechnology
α -Tubulin	Alpha-tubulin	Rabbit polyclonal	1/1,000	Abcam
MEC-17	Alpha-tubulin N-acetyltransferase 1 (MEC-17)	Rabbit polyclonal	1/500	Abcam
GSK3 β	Glycogen synthase kinase3 α/β (GSK3 α/β)	Mouse monoclonal	1/1,000	Santa Cruz Biotechnology

phospho-GSK3 β	GSK3 α/β phosphorylated at Ser9/Ser21	Rabbit polyclonal	1/500	Cell Signalling Technology
Akt	Total Akt	Rabbit polyclonal	1/1,000	Cell Signalling Technology
phospho-Akt	Akt phosphorylated at Ser473 (active Akt)	Rabbit polyclonal	1/1,000	Cell Signalling Technology
PERK	Total (PKR)-like endoplasmic reticulum kinase (PERK)	Rabbit polyclonal	1/1,000	Cell Signalling Technology
phospho-PERK	PERK phosphorylated at Thr980	Rabbit polyclonal	1/1,000	Cell Signalling Technology
eIF2 α	Total eukaryotic Initiation factor 2 (eIF2 α)	Mouse monoclonal	1/500	Cell Signalling Technology
phospho-eIF2 α	eIF2 α phosphorylated at Ser51	Rabbit polyclonal	1/500	Cell Signalling Technology
IRE1	Total inositol-requiring enzyme 1 (IRE1)	Rabbit polyclonal	1/1,000	Cell Signalling Technology
phospho-IRE1	IRE1 phosphorylated at Ser724	Rabbit polyclonal	1/1,000	Novus Biologicals
CHOP	amino acids 1-168 of full length mouse	mouse monoclonal	1/200	Santa Cruz Biotechnology
Flotillin-1	Flotillin-1	Mouse monoclonal	WB 1/500	Santa Cruz Biotechnology
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Mouse monoclonal	WB 1/2,000	Santa Cruz Biotechnology

Secondary antibodies

Antibody	Specificity	Species	Dilution	Source
AlexaFluor 680	Anti-mouse immunoglobulin G (IgG)	Goat	1/10,000	Life Technologies
IRDye 800	Anti-rabbit IgG	Goat	1/10,000	Rockland Inc
AlexaFluor 488	Anti-rabbit IgG	Goat	1/500	Life Technologies
AlexaFluor 568	Anti-mouse IgG	Goat	1/500	Life Technologies

E.coli culture

LB	20 g/L Luria-Bertani (LB) powder sterilised by autoclaving
LB agar	32 g/L agar sterilised by autoclaving 100 µg/ml Ampicillin

Plasmid DNA preparation

Plasmid DNA was prepared using a QIAprep Maxiprep Kit (Qiagen).

2.2 Methods

Culture of CHO cells

CHO cells were maintained as monolayer cultures in normal Ham's F-12 medium at 37°C under an atmosphere of 5% CO₂. Cells were passaged when CHO cells reached 70-80% confluence. After rinsing with HBSS without Ca²⁺/Mg²⁺, cells were detached using trypsin and transferred to flasks or plates with fresh culture medium, as required.

Transient transfection of CHO cells

CHO cells were transfected using jetPEI® reagent (Polyplus-transfection), according to the manufacturer's instructions, following optimisation of cell seeding density, and the amount of DNA and jetPEI in the transfection complex 24 h before transfection.

For cell counting, 10 µl cell suspension was mixed with 90 µl Trypan blue and mixed gently. 10 µl of the cell-Trypan blue mixture was loaded onto a haemocytometer. Cell

density was calculated according to equation below:

$$\text{Cell density} = \frac{\text{total cell count}}{\text{squares counted}} \times 10^5/\text{ml}$$

For most transfections using a single plasmid, 150,000 cells/well were plated into a 12-well plate, 24 h before transfection, with the culture medium being replaced with 1ml fresh Ham's F-12 immediately before transfection. For each well, 2 µg DNA was diluted with 150 mM NaCl to a final volume of 50 µl, and 2 µl jetPEI was diluted with 150 mM NaCl to a final volume of 50 µl in a separate tube. The transfection complex was made by mixing the jetPEI and DNA solutions, and incubated for 30 min at ambient temperature. The DNA/jetPEI complex was added to each well and gently swirled. Cells were harvested for biochemical assays, or fixed with 4% PFA for immunocytochemistry examination 24-48 h after transfection.

Generation of stable CHO cell lines

CHO cells were transiently transfected with plasmids encoding either wild-type full length human tau protein (FL-tau) or V5 tagged tau35 (Tau35) using jetPEI, as described above. 24 h after transfection, cells were transferred to a 25 cm² tissue culture flask. When 60% confluency was reached, the medium was replaced by selection medium (Ham's F-12 medium containing 800 µg/ml G418). Selection medium was changed every 2-3 days and, after 10-12 days, the remaining G418-resistant cells were transferred to 145 mm diameter dishes for clonal isolation. Cell clusters isolated using cloning cylinders (Sigma), were transferred to 6-well plates for clonal expansion. Further characterisation of the G418-resistant cells was undertaken using western blots to validate the expression of tau protein and immunocytochemistry to assess the homogeneity of the cell lines. Cell clones homogenously expressing 2N4R tau or tau35 (termed CHO-FL and CHO-Tau35, respectively) were selected for further experiments and maintained in Ham's F-12 medium.

Cell freezing and recovery

Cells were detached with trypsin, as described above, resuspended in Ham's F-12 medium, and centrifuged at 700 g for 5 min at ambient temperature. The cell pellet was resuspended in ice-cold cell freezing medium and dispensed by 1 ml into each cryogenic storage vial, which is then placed in an isopropanol chamber, stored overnight at -80°C and then transferred to liquid nitrogen for long-term storage.

To thaw frozen cells, cells were removed from liquid nitrogen and immediately placed into a 37 °C water bath. Thawed cells were then rapidly transferred to 75 cm² tissue culture flasks with pre-warmed Ham's F-12 medium. 24 h later, the medium was replaced by Ham's F12 to remove the DMSO.

Cell treatments

24 h before treatment, CHO-FL, CHO-Tau35 and normal CHO cells were seeded. Typically, for 6-well plates, cells were plated with a density of 300,000 cells per well, 150,000 cells per well for 12-well plates, and 80,000 cells per well for 24-well plates.

For insulin treatment, Ham's F-12 medium was replaced with medium containing 10 nM insulin, cells were treated for 30 min at 37 °C and then washed with pre-warmed PBS (×3). For LiCl treatment, cells were treated at 37 °C for 24 h with either 5 mM LiCl, or 5 mM NaCl (controls). To modulate autophagy, cells were either treated with 5 mM NH₄Cl for 24 h., or medium was replaced with HBSS (with Ca²⁺ and Mg²⁺) for 3 h. For ER stress induction, cells were incubated with either 1,4-Dithiothreitol (DTT) or thapsigargin (Fisher Scientific) at the indicated concentration for the indicated time. Cells incubated in Ham's F-12 medium were used as controls.

After treatment, cells were either lysed in 2× Laemmli sample buffer or processed according to the required experimental protocols, including Live/Dead assay, *In situ* microtubule binding assay, or immunocytochemistry.

Preparation of cell lysates

After removal of medium, cells were washed with ice-cold PBS and scraped into 2× Laemmli sample buffer (100 µl for each well of a 6-well plate). Cell lysates were diluted with 100 µl TBS containing protease and phosphatases inhibitors and heated at 95 °C for 10 min prior to analysis on SDS-PAGE.

***In situ* microtubule binding assay**

24 h after transfection, cells were rinsed with warm PBS buffer and suspended in 500µl warm microtubule-stabilising buffer. Cell lysates were centrifuged at 5,000 g for 10 min at ambient temperature, and an aliquot of the supernatant was retained (total). The remaining post-nuclear lysate was centrifuged at 100,000 g for 1 h at 37 °C. The supernatant (unbound fraction) was collected, and the pellet (bound

fraction) was rinsed twice in microtubule-stabilising buffer, pelleted at 100,000 g, then resuspended in microtubule-stabilising buffer, and sonicated briefly. All the fractions were suspended in 2× Laemmli sample buffer, heated at 95 °C for 10 min and centrifuged at 13,000 g for 5 min before analysis on SDS-PAGE.

Membrane-associated protein fractionation

24 h before harvesting, CHO-FL and CHO-Tau35 cells were plated into 10 cm diameter culture dishes. Cells were scraped into 500 µl hypotonic buffer, disrupted by sonication on ice, and centrifuged at 720 g for 10 min at 4 °C to remove unbroken cells. The resultant supernatants were centrifuged at 100,000 g for 1 h at 4 °C to produce the final supernatant (cytosolic fraction) and pellet (membrane fraction). All fractions were suspended in 2× Laemmli sample buffer, heated at 95 °C for 10 min and centrifuged at 13,000 g for 5 min before SDS-PAGE.

Sarkosyl/Triton X-100 insoluble protein fractionation

CHO-FL and CHO-Tau35 cells were maintained in 125 cm² tissue culture flasks. Cells were trypsinised at 80% confluency, as described above, washed in PBS, and resuspended in ice-cold 500 µl H buffer. Cells were lysed by sonication (×3, power level 4), and centrifuged at 500 g, for 10 min at 4 °C. The pellet, containing intact cells and nuclei, was removed, and the supernatant was centrifuged at 100,000 g for 1 h at 4 °C. The 100,000 g supernatant was retained (soluble fraction) and the pellet was resuspended in 500 µl S buffer containing 1% (v/v) Sarkosyl or Triton X-100 and incubated overnight at 4 °C. The resuspended pellet in S buffer was centrifuged at 100,000 g for 1 h at 4 °C, resulting in a Triton-soluble supernatant and a Triton-insoluble pellet. All fractions were mixed in 2× Laemmli sample buffer, heated at 95 °C for 5 min and centrifuged at 13,000 g for 10 min at 4 °C before analysis on SDS-PAGE.

SDS-PAGE & Western blots

Samples were loaded onto 10% (w/v) or 15% (w/v) polyacrylamide gels and proteins were separated by electrophoresis at 150 V until the dye front just ran off the gel.

After SDS-PAGE, separated proteins were transferred from gels onto 0.45 µm nitrocellulose membrane using a Bio-Rad wet transfer system. Sponges, filter papers, acrylamide gels, and nitrocellulose were soaked in cold transfer buffer and then assembled in the blotting cassettes as follows:

Cathode
Sponge
Filter Papers
Gel
Nitrocellulose membrane
Filter Papers
Sponge
Anode

Blotting cassettes were, immersed in transfer buffer in the blotting module and electroblotted at 100 V for 60 min, or at 30 V for 900 min, with ice cooling.

Membranes were incubated in TBS-T with 3% (w/v) milk, 5% (w/v) BSA or Odyssey blocking buffer, depending on the antibody, for 1 h at ambient temperature, to reduce the non-specific binding of antibodies. Blocked membranes were incubated with primary antibody overnight at 4 °C. Membranes were washed in TBS-T (×3) and incubated in secondary antibody for 1 h at ambient temperature before being washed again in TBS (×3).

Protein blots were visualised using the Odyssey Infrared Imaging System (Li-Cor Bioscience). Images within the linear range were captured and scanned in high resolution format. Images were analysed using Li-Cor Image Studio Lite software. After background subtraction, the bands of interest were defined manually, and the intensities were measured automatically. Signal intensities were normalised to the corresponding loading control (actin or GAPDH). Protein modifications (phosphorylation or acetylation) were determined as the ratio of the value of the modified protein to total protein. For multiple blot comparisons, a reference sample was included for standardisation between blots.

Immunocytochemistry

Cells on coverslips were washed 3 times in PBS, fixed for 10 min in 4% (w/v) PFA at 37 °C or in cold methanol (-20 °C) for 10 min then washed again in PBS. PFA-fixed cells were permeabilised using 0.25% (v/v) Triton X-100 for 10 min at ambient temperature and washed in PBS. Following incubation in blocking buffer at ambient temperature for 30 min, cells were incubated in primary antibody overnight at 4 °C. After washing 3 times in PBS, cells were incubated with secondary antibody for 1 h

at ambient temperature in the dark, followed by another 3 washes in PBS. Nuclei were stained by incubating for 1 min in Hoechst 33342 solution diluted in PBS. Coverslips were mounted on to glass slides using mounting medium (Dako, Cambridgeshire, UK) and stored in the dark. Cells were observed using a fluorescence microscope (Leica DM500B), 488 nm, 568 nm and 461 nm), adjusting emission intensity, gain and exposure time according to signal strength. Images were processed and analysed using ImageJ (<https://imagej.nih.gov/ij/>).

DNA transformation

Library Efficiency® DH5α™ competent cells (Invitrogen) were used for DNA transformation, according to the manufacturer's protocol.

Plasmid extraction

Single colonies or samples of glycerol stocks were cultivated overnight in 200 ml autoclaved LB medium, containing either 100 µg/mL ampicillin or 50 µg/mL kanamycin, at 37 °C with shaking at 220 rpm. Plasmids were prepared using the Plasmid Maxi Kit (Qiagen), according to the manufacturer's protocol. The concentration and purity of the DNA were determined by measuring absorbance at 260 nm and 280 nm (Nanodrop™ UV-Vis spectrophotometer).

Data analysis

Statistical analyses were performed using Microsoft Excel and GraphPad Prism 6. Values quantified from western blots were plotted either as a proportion of the indicated protein, or as a fold change, relative to the mean value of the appropriate controls, as indicated in the figure legends. Data were analysed using Student t-test, one-way analysis of variance (ANOVA), or two-way ANOVA, as indicated in the figure legends. Differences were taken as statistically significant if $P < 0.05$.

Chapter 3 Establishing a CHO cell model expressing Tau35

3.1 Introduction

Chinese hamster ovary (CHO) cells are one of the most commonly used mammalian cell lines used in biological and medical research. Given the advantages of CHO cells, e.g. stable biological properties, quick and easy to maintain and manipulate, and more importantly, lack of endogenous tau expression, transfected CHO cells have also been used in the field of neurodegeneration to studying physiological functions and pathological changes in tau. Using a CHO cell line stably expressing exogenous tau, Gallo et.al (1992) showed that a foetal tau isoform, containing three microtubule binding repeats, binds to microtubules and undergoes phosphorylation (Gallo et al., 1992). Stable CHO cell lines have also been used to study the different roles of various microtubule binding proteins (MAPs) in cells, such as *Drosophila* 205 kDa heat stable MAP, human MAP4, and human tau (Barlow et al., 1994). Through observing a CHO cell line stably expressing 2N4R tau, the largest isoform of human tau, it was found that expression of tau caused a change in cell shape, retarded cell growth, and dramatically altered the distribution of various organelles, including mitochondria and the endoplasmic reticulum (Ebner et al., 1998). In addition, CHO cells stably expressing tau harbouring one or more different frontotemporal lobar dementia (FTLD-tau) mutations reproduces certain disease-associated pathological features, including the formation of insoluble filamentous tau aggregates and reduced interaction of tau with microtubules. Moreover, different FTLD mutations in tau produced distinct phenotypes, demonstrating the capability of CHO cells to recapitulate diverse neurodegenerative FTLD-tau syndromes resulting from multiple mechanisms (Vogelsberg-Ragaglia et al., 2000).

Taken together, these reports show that CHO cells are a good model in which to examine the impact of disease-related changes in tau on its physiological function, especially its ability to interact with microtubules. Also, CHO cells have biochemical mechanisms capable of generating the complex features of tau related pathology, including phosphorylation and aggregation of tau.

3.2 Results

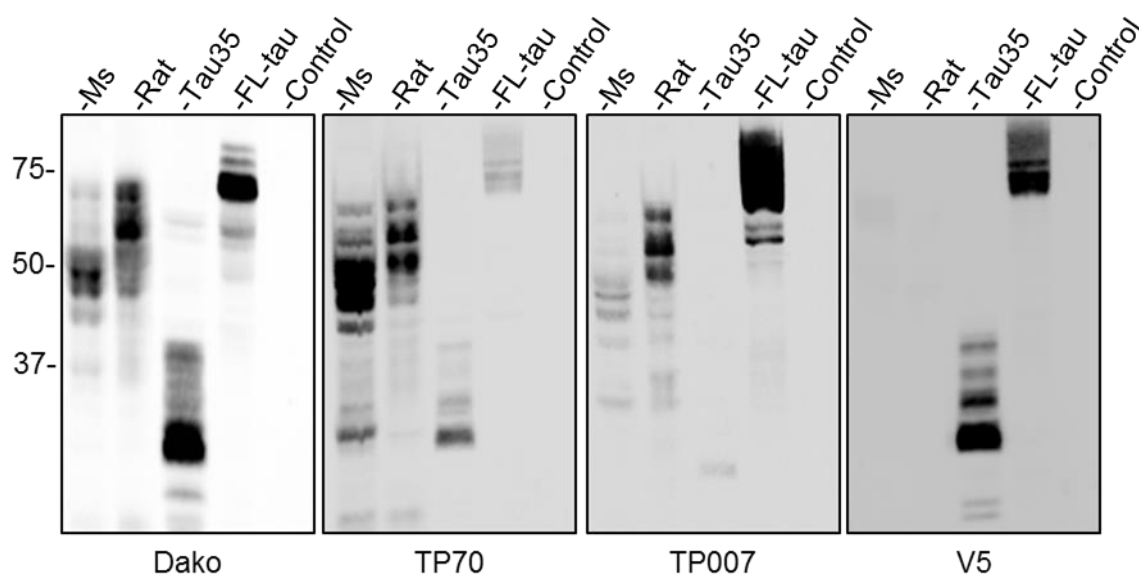
3.2.1 Profile of tau transiently expressed in CHO cells

A range of different tau antibodies were used to detect Tau35 and 2N4R Tau (FL-tau) expressed in CHO cells. CHO cells transiently transfected with plasmids encoding V5 tagged-2N4R tau or V5 tagged-Tau35 were lysed 24 h after transfection and analysed on western blots. Wild-type mouse and rat brain homogenates were used as positive controls, and untransfected CHO cells were used as a negative control.

Western blots of CHO cell lysates were probed with an antibody recognising total tau, antibodies targeting the C-terminus (TP70) or N-terminus (TP007) of tau, and an antibody recognising the V5 tag (V5). Tau from adult mouse brain was resolved into several bands corresponding to sizes of ~45-55 kDa (Fig. 3.1). Rat tau showed a broader range of bands between ~45-70 kDa, revealing differences between the tau expressed and/or post-translationally modified in these two rodent species. Both rat and mouse tau was labelled with antibodies recognising total tau and the N- and C-termini of tau.

A tau doublet corresponding to the expected molecular weight of Tau35 expressed in CHO cells was labelled by the total tau antibody, TP70 and an antibody recognising the V5 tag, but not with TP007, which is in line with the lack of the N-terminus of tau in Tau35. The Tau35 doublet is likely to be due to phosphorylation of this tau species in CHO cells. FL-tau in CHO cells was strongly labelled as a doublet of ~70-75 kDa by TP007, the antibody to total tau, and V5. The much weaker labelling of FL-tau by TP70 is likely due to the fact that TP70 labels the extreme C-terminus of tau and this may be partially obscured by the presence of the V5 tag. No signals were observed with any of these antibodies in the negative control lane of untransfected CHO cells, demonstrating a lack of non-specific binding of these antibodies in CHO cells.

Figure 3.1 Comparison of tau profiles in mouse and rat brain tau and in CHO cells expressing Tau35 or FL-tau



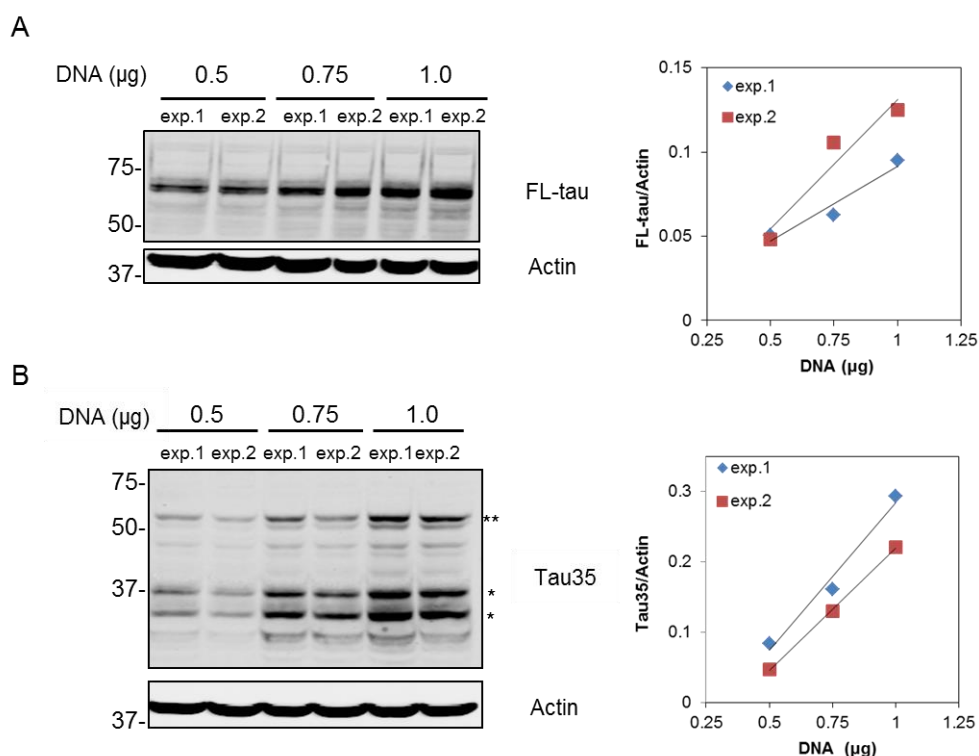
Western blots of homogenates of mouse brain (Ms), rat brain (Rat), or CHO cell lysates expressing Tau35 or full-length-tau probed with TP007, total tau, TP70, or V5 antibodies. Molecular weight markers (kDa) are shown on the left.

3.2.2 Optimisation of CHO cell transfection conditions

The first step in generating a stable cell line was to introduce a tau-expressing plasmid into CHO cells by transfection. Factors including cell density and the relative amounts of DNA and transfection reagents can dramatically affect transfection efficiency. In addition, the time gap between transfection and selection with G418 also affects the success rate of stable transfections. Therefore, the conditions for transient transfection of CHO cells were first optimised for tau expression. CHO cells were seeded at a density of 150,000 cells/well in a 12-well plate, 24 h before transfection, and transfected with 0.5 μ g, 0.75 μ g or 1 μ g of DNA, and a constant amount of 2 μ l JetPEI (Polyplus) per well, as suggested by the manufacturer. Plasmids encoding either untagged wild type full-length tau, the longest isoform of human tau, or V5-tagged Tau35, were each transfected into CHO cells. 24 h after transfection, CHO cells were lysed in 2 \times Laemmli sample buffer and immunoblotted with total tau antibody. The amount of tau expressed under each condition was

quantified relative to the amount of actin. The western blots showed bands corresponding to FL-tau at approximately 70 kDa (Fig. 3.2A), whereas Tau35, exhibited two major bands at 33 kDa and 35 kDa (indicated with *), probably due to different phosphorylation level (Fig. 3.2B). One additional tau specie at around 60 kDa is also detected (indicated with **). A possible explanation for the appearance of these higher molecular weight bands in the Tau35-expressing CHO cells is the formation of Tau35 oligomers. The transfection efficiency of both FL-tau and Tau35 plasmids increased in a DNA-dependent manner. Doubling the amount of DNA resulted in a one-fold increase in the expression of FL-tau and three-folds increase of Tau35 (Fig. 3.2C, D). It is notable that the expression of Tau35 was relatively higher than that of FL-tau, possibly due to the difference in the sized between two plasmids. Meanwhile, the signal of actin within each experiment showed no difference, indicating none of the conditions is cytotoxic. Therefore, to obtain the highest expression level of these two proteins without causing cell toxicity, 1 µg DNA was used for transient transfection.

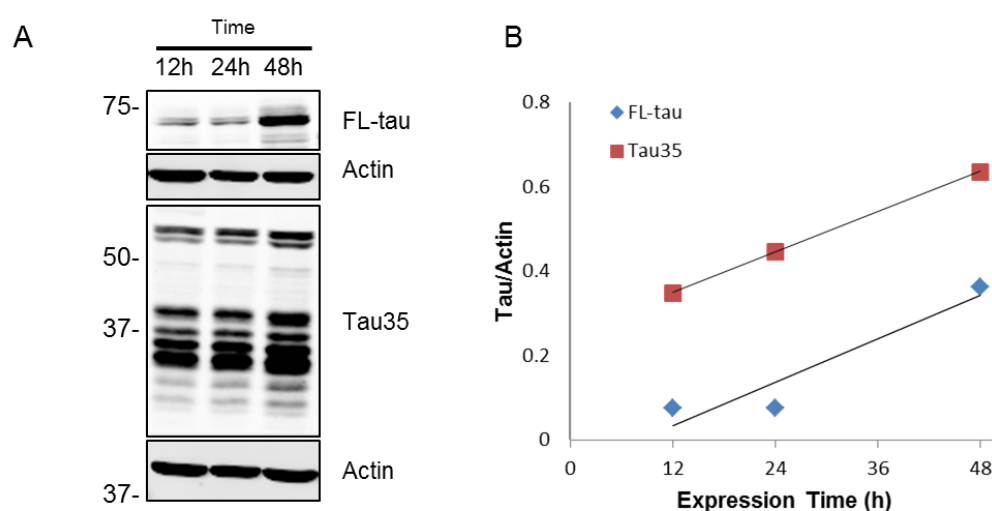
Figure 3.2 Transfection efficiency of tau constructs increased in a DNA-dependent manner



Western blots of lysates of CHO cells transfected with 0.5, 0.75, or 1 µg of DNA encoding either (A) FL-tau or (B) Tau35 and probed with antibodies to total tau and β -actin (Actin). Molecular weight markers (kDa) are shown on the left. Different Tau35 bands are indicated with *. Two bands around 37 kDa are indicated with *, and the band at 60 kDa is indicated with **. Graphs show the amount of full-length tau or Tau35 protein expressed, relative to β -actin. Data was obtained from 2 independent experiments (exp.1 and exp. 2).

To assess the changes in the expression of tau protein over time, cells were harvested 12, 24, and 48 h after transfection. The abundance of FL-tau and Tau35 was analysed on western blots, standardised to β -actin. The expression level of both FL-tau and Tau35 both increased over time up to 48 h after transfection (Figure 3.3).

Figure 3.3 Exogenous expression of tau in CHO cells increased in a time-dependent manner



(A) Western blots of CHO cells expressing FL-tau or Tau35 harvested 12, 24, or 48 h post-transfection and probed with antibodies to total tau and β -actin (Actin). Molecular weight markers (kDa) are shown on the left. (B) Graphs showing the amount of FL-tau or Tau35 relative to β -actin expressed in CHO cells over time. Data were obtained from one experiment.

3.2.3 Selection and validation of colonies of CHO cells expressing FL-tau or Tau35

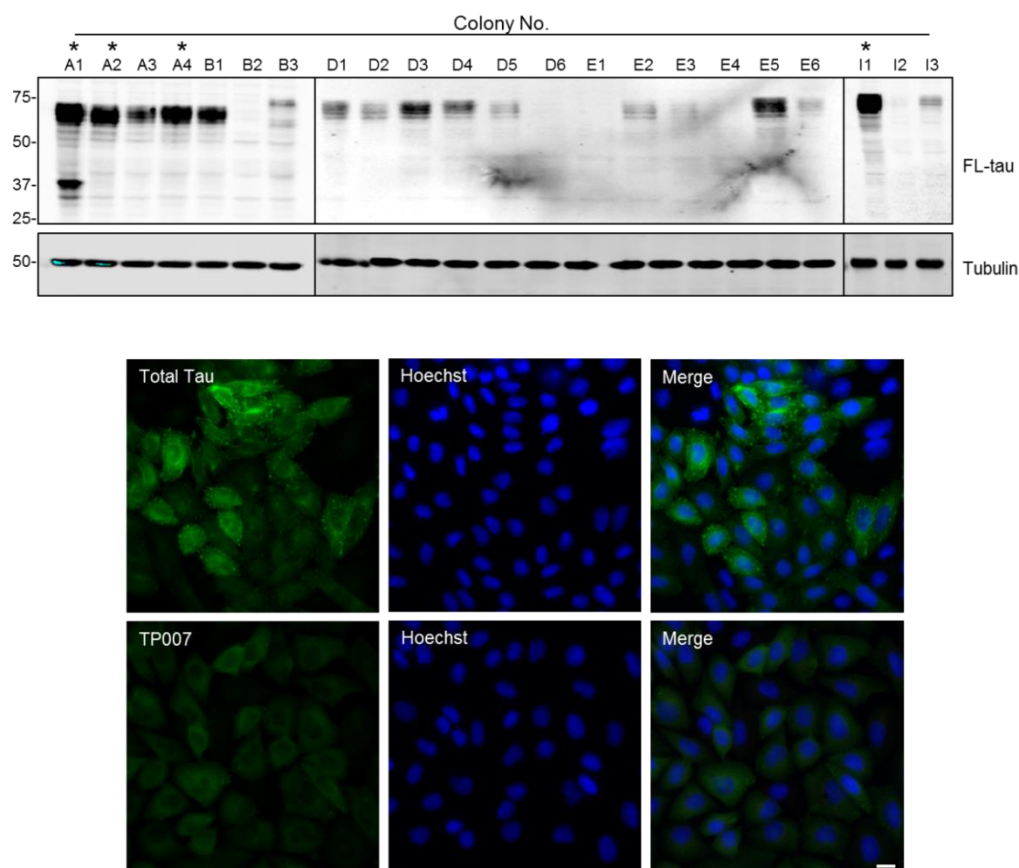
CHO cells expressing FL-tau or Tau35 were selected on the basis of G418 resistance. According to previous literature wherein CHO cell lines stably expressing tau mutants are employed (Vogelsberg-Ragaglia et al., 2000), 800 µg/ml G418 was used to select tau-expressing cells and untransfected CHO cells were included as a positive control G418 selection only of cells harbouring the tau-expressing plasmid (Ebner et al., 1998). Cell mixture expressing FL-Tau/Tau35 were first generated through transient transfection, according to the conditions determined above (Section 3.2 and 3.3). G418 selection was carried out 48 h after transfection. After 12 days of G418 selection, all of the untransfected CHO cells had died, since they lack resistance to this antibiotic. On day 18 G418-resistant colonies of transfected CHO cells were diluted to 0.8 cell/well by limiting dilution and plated on 96-well plates to isolate individual monoclonal colonies. Clonal cells were maintained in selection medium, amplified and analysed on western blots. The homogeneity of the tau-expressing cells was determined by immunofluorescence using tau antibodies.

For FL-tau, 22 colonies were isolated during the first round of cloning, and western blots were used to show that 15 of these clones expressed FL-tau (Fig. 3.4A). Four colonies (A1, A2, A4, and I1, indicated with asterisks) were selected as those exhibiting the highest apparent expression of FL-tau. Of the remaining 20 colonies, seven showed moderate expression of tau (A3, B1, D1, D2, D3, D4, and E5), three displayed low levels of expression (D5, E2 and I3) and six did not express tau (B2, D6, E1, E3, E4, I2). Notably, for colonies expressing FL-tau, colony A1 displayed high expression of FL-tau, whereas generated a tau fragment at 38kDa.

Homogeneity of colonies were confirmed by immunofluorescence labelling. Cells were fixed with 4% paraformaldehyde (PFA) which preserves most of the cytosolic proteins and labelled with total tau antibody. Of the four highest expressing tau colonies, determined by western blot (indicated by asterisks in Fig. 3.4), A1 was excluded due to the presence of an unknown tau fragment of ~38 kDa. The remaining three colonies (A2, A4 and I1) were fixed with 4% (w/v) PFA, and examined by immunofluorescent labelling with total tau antibody. Colonies A2 and A4 both exhibited heterogeneous tau expression and therefore colony I1 was selected for further studies. In contrast, colony I1 showed that ~95% of the cells are tau positive (Fig. 3.4B). TP007 was also used to ensure the tau species expressed

in colony I1 included the N-terminus of tau. Extensive tau labelling was found in the cytoplasm but not the nuclei of CHO cells, indicating FL-tau is mostly cytosolic protein. However, given the high expression level of FL-tau as well as the limitation of PFA fixation, the exact localisation of FL-tau as well as its interaction with microtubules is yet to be looked into. Moreover, dense dots with high levels of tau immunoreactivity were detected. The presence of these dots can be attributed to several possible reasons, including tau aggregation, formation of vesicles containing tau, and accumulation of tau within certain organelles, whereas more thorough investigations are needed to verify such possibilities.

Figure 3.4 Selection and validation of G418 resistant colonies expressing FL-tau



(A) Western blot analysis of cell lysates of individual colonies of CHO cells expressing FL-tau, labelled with antibodies to total tau and α -tubulin (Tubulin). Candidate colonies are indicated with *. Molecular weight markers (kDa) are shown on the left. (B) Immunofluorescence of CHO cells expressing FL-tau, colony I1. Cells were fixed with 4% paraformaldehyde (PFA) and labelled with antibody to total tau (green, upper panel), TP007 (green, lower panel) and Hoescht 33342 to label nuclei (blue). The rightmost panels show the merged images. Scale bar=10 μ m.

All the 14 tau expressing colonies, including high, moderate and low expressers were then preserved by freezing and subsequent storage in liquid nitrogen. Colony I1 was thawed after one week of freezing, to assess cell viability and to further characterise this new FL-tau-expressing CHO cell line.

Similarly, for cell lines stably expressing Tau35, 11 colonies were isolated and characterised on western blots and by immunofluorescence. Ten of the 11 colonies expressed Tau35 (Fig 3.5). The band pattern of Tau35 on western blots of these colonies resembled those obtained from CHO cells transiently transfected with Tau35 labelled with total tau antibody. The cell lysates displayed an array of Tau35 species ranging from 39 kDa to 30 kDa. Of the ten Tau35 expressing colonies, colony 10 exhibited the highest Tau35 expression, relative to tubulin (Fig. 3.5). Immunofluorescence revealed that cells derived from colony 10 exhibited >90% tau positive staining. This colony was then preserved by freezing and subsequent storage in liquid nitrogen. Similar to CHO cells expressing FL-tau, most Tau35 proteins remains in the cytoplasm and did not colocalise either with α -tubulin nor with nuclei staining, indicating Tau35 as a cytosolic protein.

Cells thawed and recovered from liquid nitrogen 14 days after freezing were subjected to another western blot analysis to confirm the stable expression of Tau35.

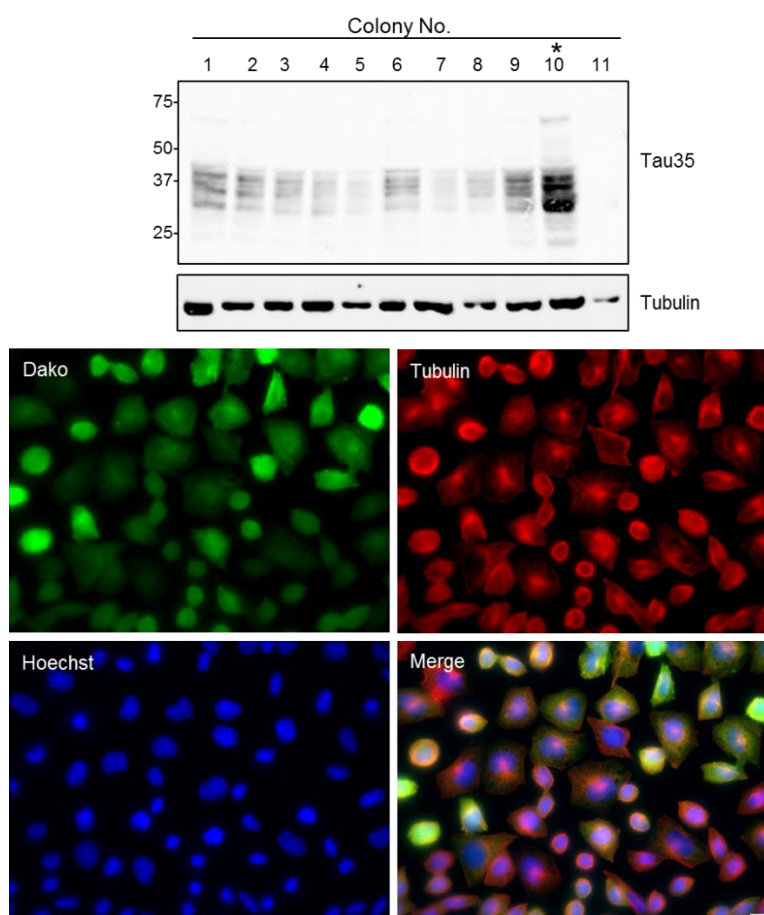
3.2.4 Verification of stable CHO cell lines expressing FL-tau or Tau35

Colonies I1 (FL-tau) and 10 (Tau35) were recovered from freezing and tested to verify whether expression of the exogenous protein, as well as the homogeneity of the cells, was unchanged following a freeze-thaw cycle and successive passages in culture.

Both colonies were maintained in G418 selection medium, and split when they reached 70% confluency. During each passage, a portion of the cell culture was collected and lysed for western blot analysis. Cells passaged from colonies I1 and 10 showed equivalent expression of FL-tau and Tau35, respectively, relative to tubulin, between passages 2 to 5 (Fig 3.6A, B). Immunofluorescence analysis of colony I1 and colony 10 as passage No.5 revealed that the homogeneity of tau expression in these cells was not affected by successive passaging during this period (Fig 3.6C). Importantly, expression of FL-tau or Tau35 dramatically alters the morphology of CHO cells, this finding will be discussed in further detail in next chapter.

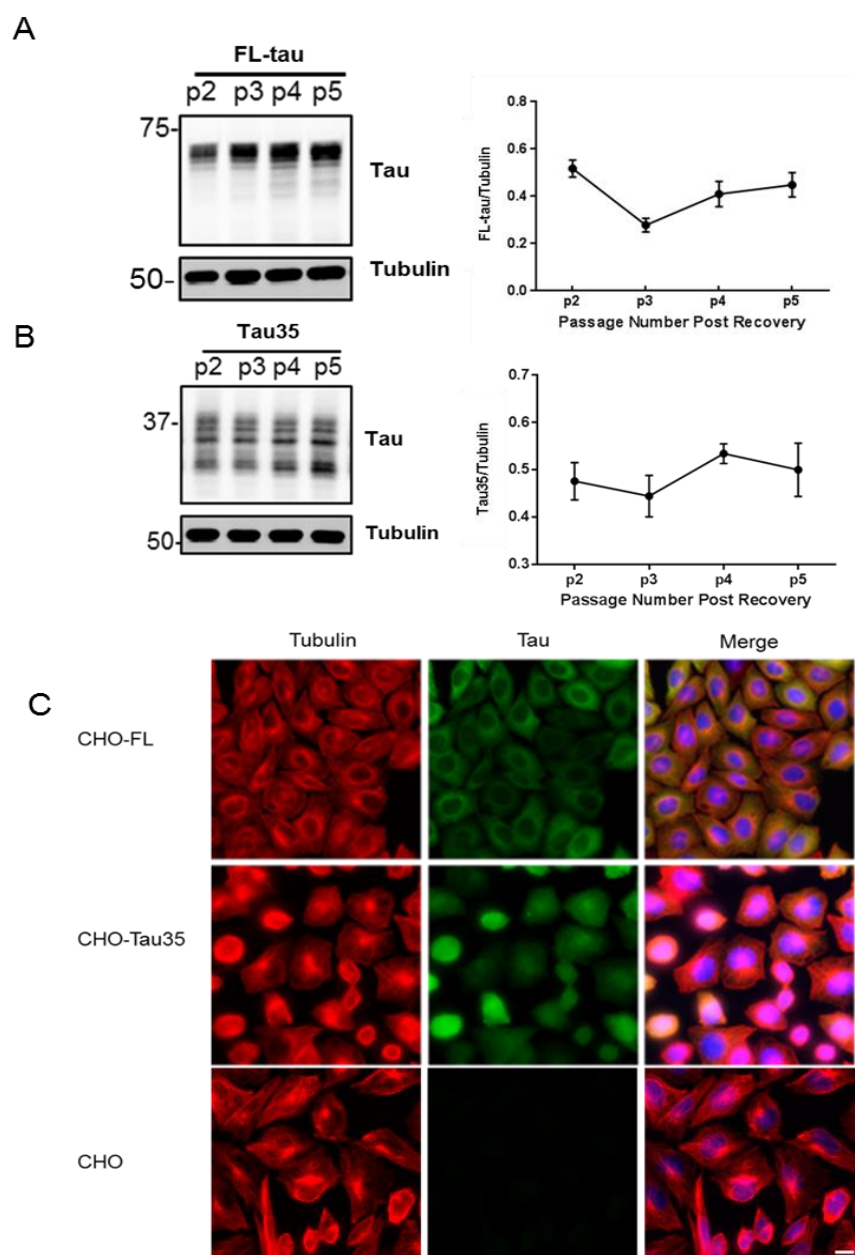
In conclusion, given the stability of the transfected plasmids in colonies I1 and 10, cell lines derived from these two colonies were selected for further experiments. Colony I1 is hereinafter thus termed CHO-FL, and colony 10 termed CHO-Tau35.

Figure 3.5 Selection and validation of G418 resistant colonies expressing Tau35



(A) Western blot analysis of cell lysates of individual colonies of CHO cells stably expressing Tau35, labelled with antibodies to total tau and α -tubulin (Tubulin). Candidate colony is indicated with *. Molecular weight markers (kDa) are shown on the left. (B) Immunofluorescence of CHO cells expressing Tau35, colony 10, labelled with antibody to total tau (green), α -tubulin (red), and Hoescht 33342 (blue). The lower right panel show the merged images. Scale bar=10 μ m.

Figure 3.6 Verification of the stability of expression of FL-tau and Tau35 in stable CHO cell lines



(A) Western blot analysis of CHO cell lysates of FL tau and Tau35 expressing lines (colonies I1 and 0, respectively) from passage numbers 2 to 5, probed with antibodies to total tau and α -tubulin (Tubulin). (B) Graphs showing the amount of FL-tau or Tau35 relative to α -tubulin expressed in CHO cell colonies I1 and 10 over successive passages. Values represent mean \pm standard error of the mean (S.E.M), $n=3$. (C) Immunofluorescence of CHO cells expressing FL tau and Tau35. For CHO-FL and CHO-Tau35, images were acquired at passage 5. Antibodies to total tau (green) and α -tubulin (red) were used. Nuclei were stained with Hoescht 33342 (blue). Scale bar=10 μ m.

3.3 Discussion

In this chapter, a CHO cell model stably expressing Tau35, the pathology related tau fragment derived from human brain of tauopathies, was established. A CHO cell line stably expressing FL-tau, the longest isoform of human tau in the CNS, was also generated as a control for the effects of tau expression in CHO cells.

During optimisation of the conditions for transient transfection, transfection efficiency was found to increase in a DNA-dependent manner. Furthermore, expression of the target proteins in transiently transfected cells increased in a time-dependent manner.

Use of G418, enabled selection of transfected from non-transfected cells and several CHO cell clonal colonies were isolated expressing FL-tau or Tau35. However, western blot analysis revealed that not all of the isolated colonies expressed tau, even though they were G418 resistant. This artifact may be caused by incomplete insertion of the exogenous plasmid into the genome of CHO cells. Further experiments are still needed to identify the relationship between the 38kDa tau fragment detected in colony A1 and full length tau.

Two CHO cell lines were selected as expressing significant amounts of FL tau or Tau35, and this was found to remain consistent throughout successive passages 2-5. Furthermore, these two cell strains were preserved and amplified into new tau-expressing CHO cell lines for further research. Notably, it was later observed that for CHO-FL, expression level of full-length tau protein start to decrease when the passage number reached 17, whereas decreased expression of Tau35 is found in CHO-Tau35 cells after passage number reached 20.

The value of stable cell lines in studying mechanisms underlying tau-related cell death has been shown in several studies (Barlow et al., 1994; Matsumura et al., 1999; Vogelsberg-Ragaglia et al., 2000). This new CHO cell model stably expressing a disease-associated tau fragment will allow researchers to examine the role of tau fragmentation in the development of tauopathies. It will also serve to provide an *in vitro* model with the potential for screening of drug candidates, facilitating pharmaceutical interventions in these diseases.

Chapter 4 N-terminal cleavage of tau leads to the loss-of-function

4.1 Introduction

In addition to the well documented role of abnormal phosphorylation of tau, other tau modifications, such as proteolytic cleavage at the C-terminus, have been linked to disease pathogenesis in the tauopathies. Two well characterised tau fragments, truncated at either Asp421 or Glu391, are related to neurofibrillary pathology in AD brain, and to the gain-of-toxicity of tau which lead to cell death in primary cortical neurons (Basurto-Islas et al., 2008).

However, the impact of N-terminal cleavage on tau function and toxicity is not well understood. Evidence has indicated that the N-terminal half of tau may participate in the regulation of the tau-microtubule interactions. On the contrary, Matsumoto and colleagues (Matsumoto et al., 2015) reported that N-terminal cleavage of tau at Gln124 enhanced binding of the resultant fragment to microtubules, and thereby increased tubulin stability (Derisbourg et al., 2015). However, N-terminal cleavage of tau at Arg242 resulted in a loss of the ability of tau to promote microtubule assembly and loss of the capacity of tau to bundle microtubules in cultured cells. *In vitro* studies showed that tau is prone to adopt a “paperclip” superstructure whereby the two ends of the molecule approach each other and the repeat domain (Fig 1.3), providing evidence of the involvement of the N-terminal half of tau in the microtubule interactions (Carmel et al., 1996; Jeganathan et al., 2006). However, contradictory results showing N-terminally truncated tau species starting at residue Gln124 display a stronger ability to bind and stabilise microtubules, imply that further experiments are still needed to fully elaborate the role of the N-terminal half of tau in mediating its interactions with microtubules (Derisbourg et al., 2015). Therefore, investigating how the physiological properties of tau are changed upon truncation will shed new light on the importance of the role of the N-terminal region. This knowledge will also aid understanding of how abnormal cleavage of tau contributes to the progression of tau pathology in the tauopathies.

This chapter describes a series of functional studies analysing the biochemical characteristics of full-length tau and comparing the results with those obtained with Tau35, which lacks the N-terminal half of the molecule.

4.2 Results

4.2.1 Expression of full-length tau or Tau35 alters the morphology of CHO cells

Stable cell lines generated previously, namely CHO-FL, CHO-Tau35 (Chapter 3 Section 3) and untransfected CHO cells, were fixed with 4% PFA and probed with antibodies against tau and α -tubulin to investigate changes in morphology resulting from tau expression. CHO cells normally exhibit a spindle-like appearance in culture, with a clear microtubule cytoskeleton (Fig 4.1, upper panels). In contrast, exogenous expression of full-length tau caused the formation of a perinuclear microtubule bundles in CHO cells, and due to the expansion of the microtubule skeleton, the cells acquired a more rounded morphology (Fig. 4.1, middle panels). The tau expressed in CHO-FL cells was associated with the microtubules and was absent from the nucleus. CHO-Tau35 cells appeared smaller in size, more rounded, and they lacked a well-defined microtubule structure, compared to CHO-FL cells (Fig. 4.1, lower panels). Tau35 expressed in CHO-Tau35 cells was apparent throughout the cells, but a well-defined microtubule lattice is absent from the cells.

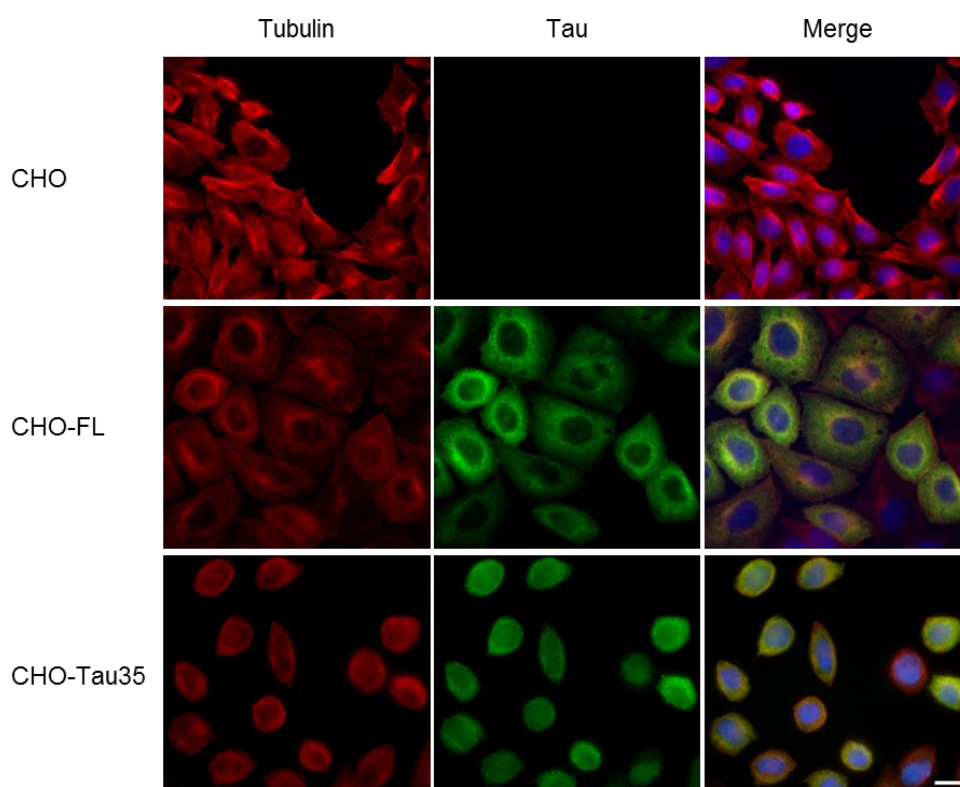
4.2.2 Tau35 displayed reduced microtubule binding ability

One of the defining functions of tau is to bind microtubules and to promote their stability. It has been proposed that in tauopathy brain, loss of this function may result in the breakdown of axonal transport of vesicles and organelles, leading to neurodegeneration. To examine the effects of N-terminal cleavage on the ability of tau to bind microtubules, an *in situ* microtubule binding assay was carried out. In CHO-FL and CHO-Tau35 cells, microtubules were stabilised with taxol in the presence of GTP, and the cellular content was separated into microtubule-bound and microtubule-unbound fractions using ultracentrifugation. The separated fractions were analysed on western blots probed with antibodies to tau and α -tubulin (Fig. 4.2). For each cell line, 1% (v/v) of the total and unbound fraction and 5% (v/v) of the bound fraction was loaded.

As shown in Figure 4.2, In CHO-FL cells, tau is present in both microtubule-bound and unbound fractions, with approximately 22% of the total amount of tau bound to microtubules (Fig. 4.2A). These findings are in line with a previous report of 17% of total tau binding to microtubules *in situ* (Rodríguez-Martín et al., 2013). However, in CHO-Tau35 cells, only a minor amount of Tau35 is present in the microtubule-bound fraction, equivalent to approximately 6% of total amount of Tau35 (Fig. 4.2A). These

results show a significant ($P<0.05$) impairment in the ability of Tau35 to bind to microtubules when expressed in CHO cells, compared to full-length tau. These results show a reduction of 66% in the microtubule binding ability of Tau35 caused by the deletion of the N-terminal half of tau, despite the presence of the microtubule-binding domain in Tau35.

Figure 4.1 Tau expression alters cell morphology

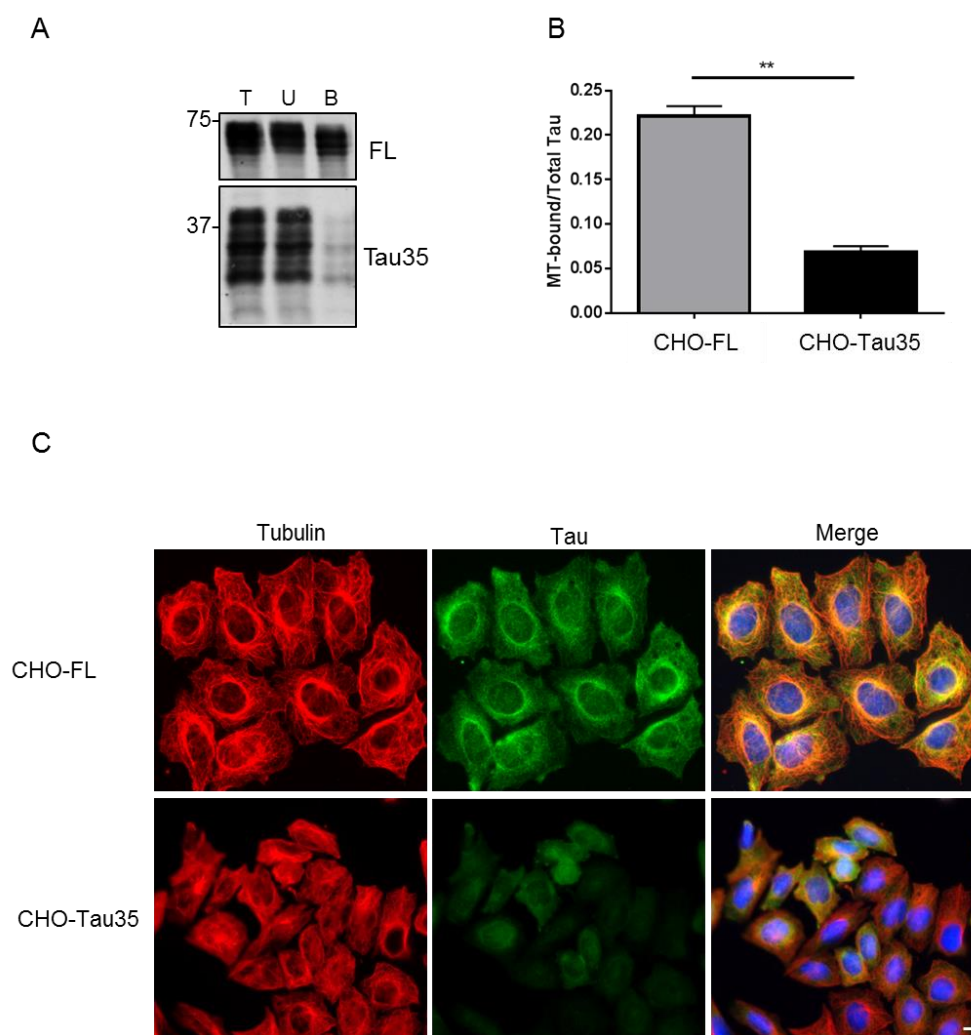


Immunofluorescence of CHO, CHO cells stably expressing full-length tau (CHO-FL) or Tau35 (CHO-Tau35), fixed with 4% PFA and labelled with antibody to α -tubulin (red, left panels), total tau (green, middle panels), and Hoescht 33342 (blue). The rightmost panels show the merged images. Scale bar=10 μ m.

Immunocytochemistry of methanol-fixed CHO cells was used to preserve and increase the visibility of the cytoskeleton. In CHO-FL cells, tau induced a circular rearrangement of the microtubule array, forming microtubule bundles, which were predominantly peri-nuclear (Fig. 4.2B). In addition, a substantial fraction of full-length tau was distributed along the microtubules and co-localised with the ring-like

microtubule bundles. However, in CHO-Tau35 cells, microtubule filaments are distributed randomly in the cells without forming well-organised structures. Moreover, unlike full-length tau, Tau35 was not bound to MTs following methanol fixation, and no significant co-localisation of Tau35 with microtubules was detectable, indicating a more cytosolic localisation of Tau35, in agreement with the results obtained by biochemical fractionation (Fig. 4.2A).

Figure 4.2 Tau35 has a reduced ability to bind to microtubules in CHO cells



(A) Western blots of total lysate (T), microtubule-unbound (U) and microtubule-bound (B) fractions of CHO-FL and CHO-Tau35 cells probed with antibody against total tau. Molecular weight markers (kDa) are shown on the left. Graph shows the ratio of tau present in the microtubule-bound fraction to tau in the total cell lysate. Values represent mean \pm S.E.M; Student t-test, n=3; **P<0.01. (B) Immunofluorescence of methanol-fixed CHO-FL and CHO-Tau35 cells, labelled with antibodies to α -tubulin (red), total tau (green) and Hoescht 33342 (blue). The rightmost panels show the merged images. Scale bar=10 μ m.

Overall the data demonstrate that although Tau35 retains an intact microtubule binding domain, the ability of Tau35 to bind to microtubules is greatly reduced by deletion of the N-terminal region. As a consequence, the morphology of the microtubule cytoskeleton is significantly altered by Tau35 expression. In the presence of Tau35, microtubules displayed a random and somewhat disorganised morphology, which was in contrast to the ring-like microtubule bundles seen in CHO-FL cells.

4.2.3 Tau35 fails to promote tubulin acetylation in contrast to FL tau

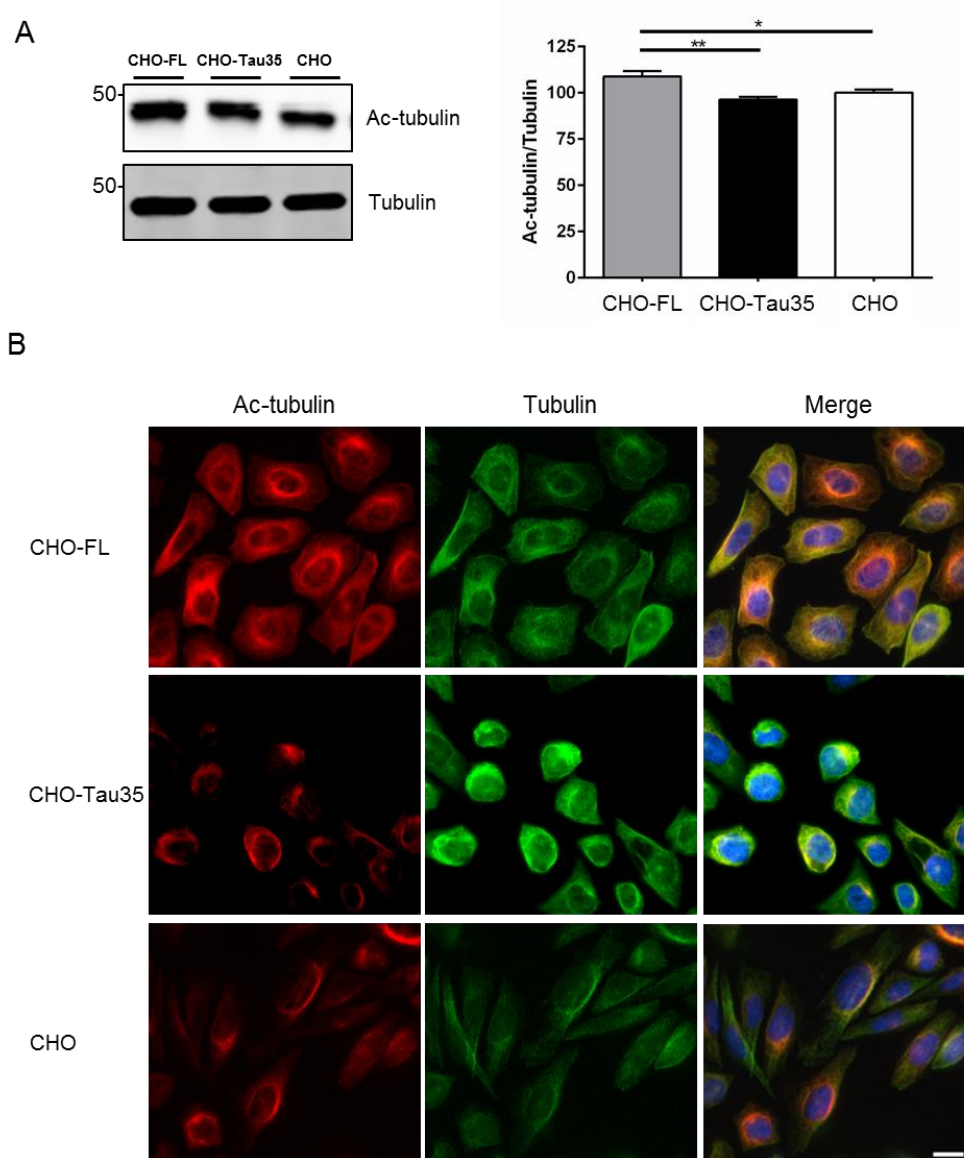
Accumulating evidence has indicated the importance of tubulin acetylation in modulating the function of microtubules (Perdiz et al., 2011). Acetylation of the Lys40 residue in α -tubulin is regarded as a marker of polymer age and hence microtubule stability (Gundersen et al., 1984; Piperno et al., 1987; Schulze et al., 1987). Correspondingly, defects in tubulin acetylation have been found in a variety of neurodegenerative disorders, such as Huntington's disease, Parkinson's disease and tauopathies (Dubey et al., 2015; Zhang et al., 2015).

To determine the impact of Tau35 on tubulin acetylation and tubulin expression, cell lysates of each CHO cell line were analysed on western blots probed with antibodies against α -tubulin acetylated at K40 (Ac-tubulin) and total α -tubulin. CHO-FL cells exhibited a 9% increase ($P < 0.05$) and CHO-Tau35 cells a slight decrease (4%, $P > 0.05$) in tubulin acetylation, compared to normal CHO cells (Fig. 4.3A). These results indicate a potential increase in tubulin stability in CHO-FL which is absent in CHO-Tau35 cells in comparison to CHO cells.

Immunocytochemical analysis of PFA-fixed cells further showed that, with the same exposure time and excitation intensity, CHO-FL cells displayed a higher intensity of acetylated α -tubulin signal to CHO-Tau35 and CHO cells (Fig. 4.3B). Both acetylated tubulin and tubulin are more filamentous in CHO-FL cells. In contrast, tubulin in CHO-Tau35 cells was more diffuse, with a lower extent of organisation. In CHO-FL cells, the overlap between total tubulin and acetylated tubulin can be observed extensively across the cell body, indicative of increased acetylation, whereas in CHO-Tau35 and CHO cells, acetylated tubulin was mainly distributed in the tubulin subpopulation around nuclei. In contrast to CHO-FL cells, in CHO-Tau35 cells, although some microtubules are acetylation-positive, the majority of the tubulin population was acetylation negative. These data suggest that Tau35 is unable to

promote tubulin acetylation in CHO-Tau35 cells, which further supports the role of Tau35 in tubulin disorganisation.

Figure 4.3 Tau35 fails to promote tubulin acetylation



(A) Western blots of CHO-FL, CHO-Tau35 and CHO cells with antibodies against acetylated (Ac-tubulin) and total α -tubulin (Tubulin). Molecular weight markers (kDa) are shown on the left. Graph shows fold changes relative to CHO cells in the ratio of acetylated α -tubulin to total α -tubulin. Values represent mean \pm S.E.M, $n=4$, one-way ANOVA, * $P<0.05$, ** $P<0.01$. (B) Immunofluorescence of CHO-FL, CHO-Tau35, and CHO cells fixed with 4% PFA, and labelled with antibodies to acetylated α -tubulin (red, left panels), total α -tubulin (green, middle panels), and Hoechst 33342 (blue). The rightmost panels show the merged images. Scale bar=10 μ m.

4.2.4 Microtubule bundling is not restored by MEC-17

Recently, mechanosensory abnormal 17 (MEC-17), also known as α -tubulin acetyltransferase 1 (ATA1), was identified as an important α -tubulin acetyltransferase (Akella et al., 2010). MEC-17 protects neurons by stabilising the cytoskeleton, enabling transport of essential molecules and organelles, including mitochondria, through the axon (Neumann and Hilliard, 2014). Overexpression of MEC-17 has been shown to restore α -tubulin acetylation induced by leucine-rich repeat kinase 2-containing pathogenic Roc-COR domain mutations (Godena et al., 2014).

Therefore, to investigate how increased tubulin acetylation facilitates microtubule organisation in the presence of full-length tau or Tau35, CHO cells were transiently transfected with a plasmid expressing MEC-17 and examined 48 h post transfection. Immunocytochemical analysis showed that MEC-17 expression increased tubulin acetylation in each of the three cell lines (Fig. 4.4A). Microtubule formation was significantly enhanced in CHO-FL cells. Thick microtubule bundles formed of highly acetylated tubulin filaments was detected widely across the CHO-FL cell population. In CHO-Tau35 cells, increasing tubulin acetylation resulted in limited microtubule bundling, but a random accumulation of tubulin filaments. No obvious phenotypical changes were detected in CHO cells expressing MEC-17. Expression of MEC-17 in the transfected cell lines was confirmed on western blots probed with MEC-17 antibody (Fig. 4.4B).

The numbers of microtubule bundles present in each cell type were determined in the presence and absence of exogenous MEC-17 expression. The results showed that before transfection, the percentage of cells harbouring microtubule bundles over total cells was 20%, 11%, and 17% ($P > 0.05$) in CHO-FL, CHO-Tau35 and CHO cells, respectively (Fig. 4.4C). While after transfection, MEC-17 expression increased the percentage of microtubule bundle-bearing cells relative to total cell number to 40%, 20%, and 24% in CHO-FL, CHO-Tau35 and CHO cells, respectively. The MEC-17-induced increase in microtubule bundles was approximately two-fold in both CHO-FL and CHO-Tau35 cells ($P < 0.05$), but the apparent increase in bundles in CHO cells lacking tau was not statistically significant. Notably however, CHO-Tau35 cells behave similarly to untransfected CHO cells in terms of tubulin acetylation, indicating a potential loss-of-function in the CHO-Tau35 cells with respect to tubulin acetylation.

Figure 4.4 MEC-17 is less effective in facilitating microtubule bundling in CHO-Tau35 cells

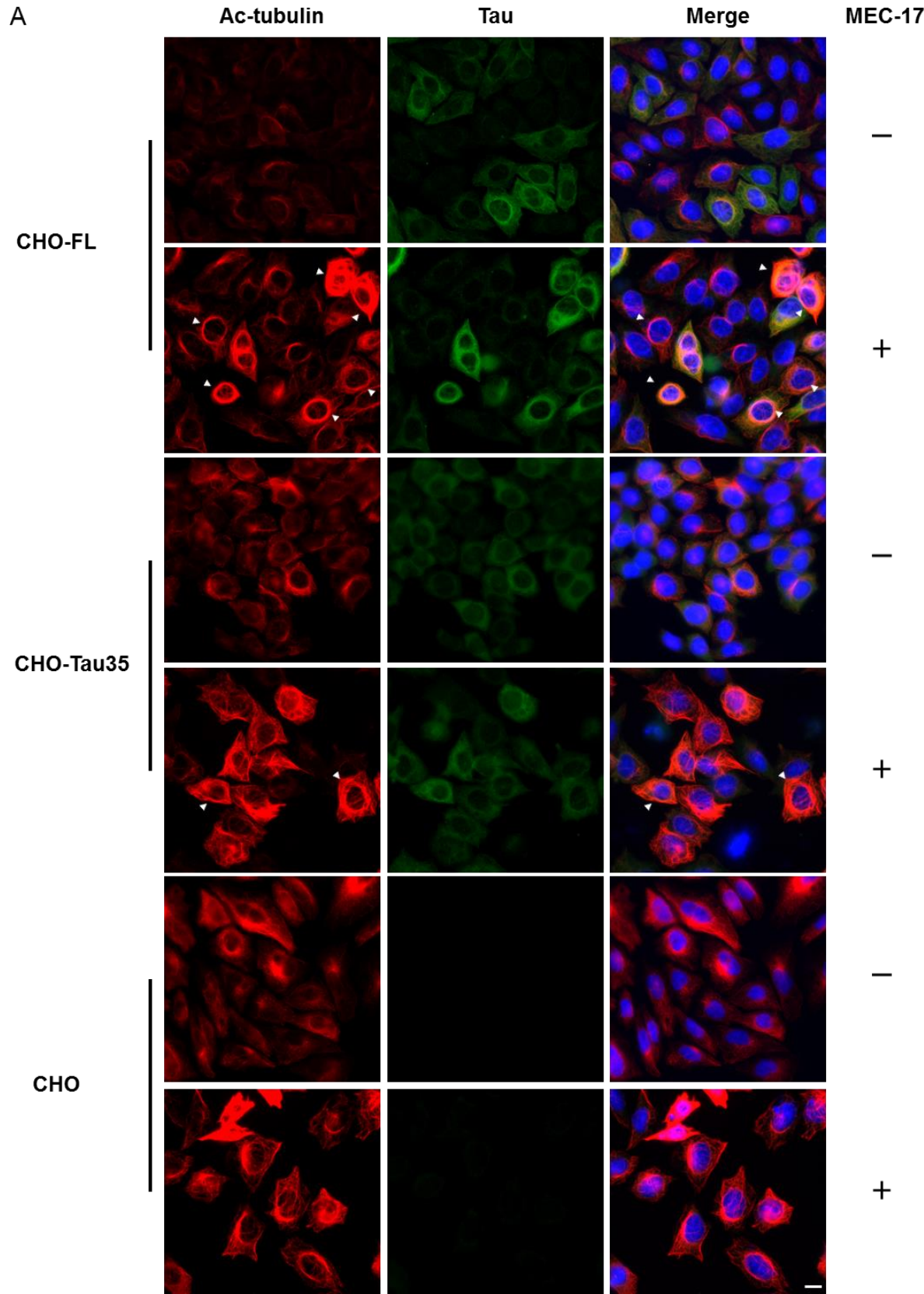
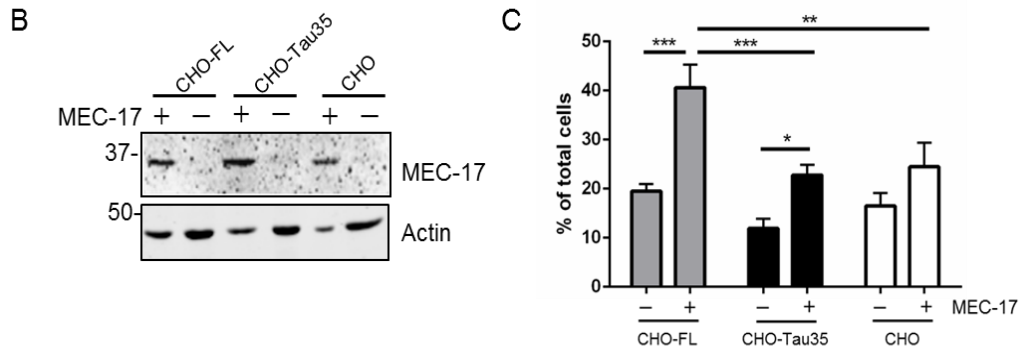


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(A) CHO-FL, CHO-Tau35 and CHO cells were transiently transfected with a plasmid expressing MEC-17 (+) or without plasmid (-). Immunofluorescence of methanol-fixed CHO cells labelled with antibodies to acetylated α -tubulin (red), and total tau (green), and Hoechst 33342 (blue). The rightmost panels show the merged images. Arrowheads indicate cells bearing MT bundles. Scale bar=10 μ m. (B) Western blots probed with antibodies recognising MEC-17 and β -actin. Molecular weights (kDa) are shown on the left. (C) Quantification of microtubule bundles in CHO cells. For each condition, a total number of ≥ 150 cells were counted. Values represent mean \pm S.E.M; two-way ANOVA, * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$.

It was also notable that, unlike CHO-FL cells, in which cytoskeletal-bound tau is readily observed, Tau35 failed to co-localise with microtubule filaments, despite the changes in induced by Tau35 expression on microtubule structure. This finding suggests that increasing tubulin acetylation is not able to recover the interaction between Tau35 and microtubules.

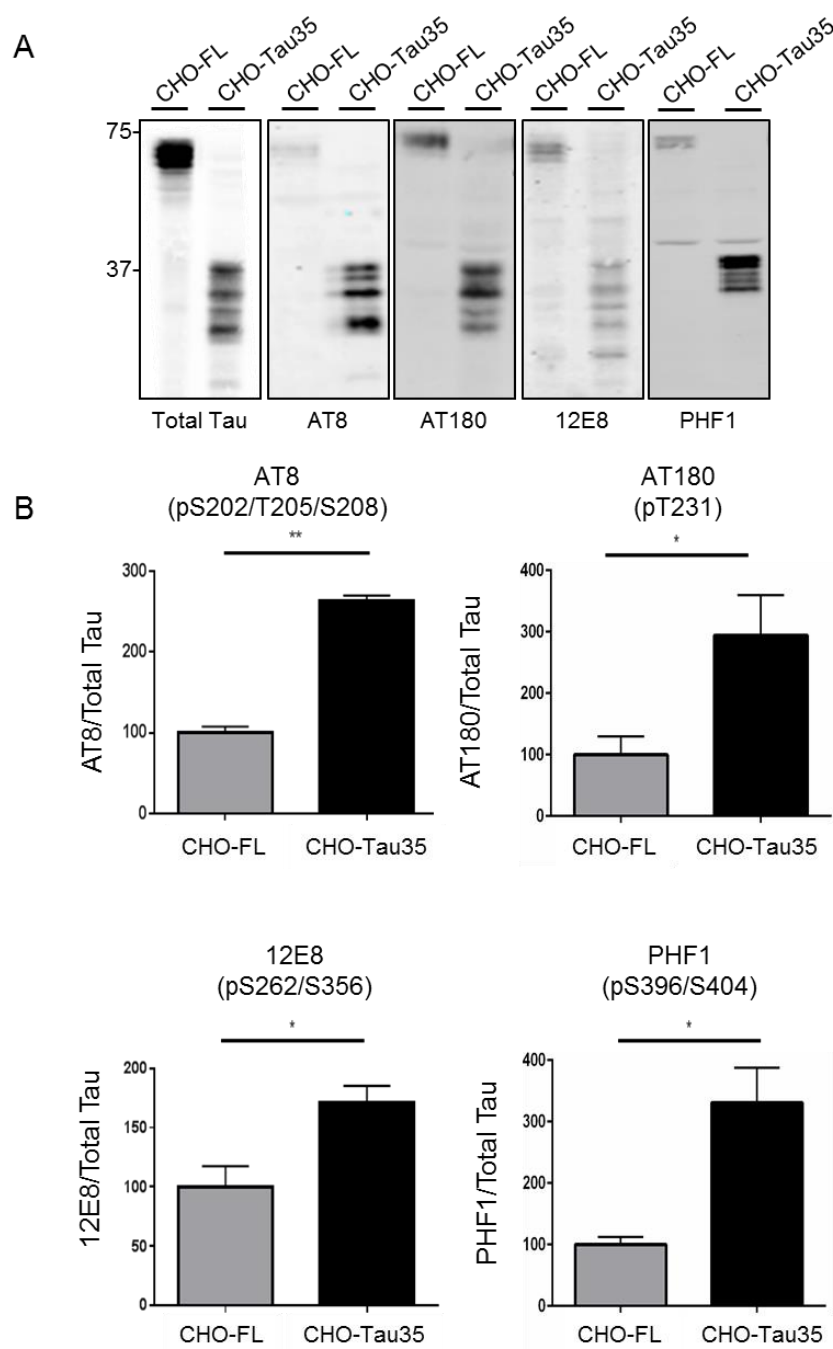
4.2.5 Tau35 is more prone to hyperphosphorylation than full-length tau

Phosphorylation plays a crucial part in regulating the physiological functions of tau, including its binding to microtubules, and therefore regulates the stabilisation and assembly of microtubules themselves. Thus the interaction of tau with microtubules is downregulated by phosphorylation at specific residues on tau, including Ser262 and Thr231 (Biernat et al., 1993; Ding et al., 2006). Meanwhile, aberrant phosphorylation at Ser396/Ser404 and Ser199/Ser202/Thr205 are reported to be associated with pathological changes in tau (Avila et al., 2012; Hanger et al., 2009; Martin et al., 2011).

Tau35 phosphorylation was evaluated on western blots probed with a series of four phospho-dependent tau antibodies targeting residues across the entire sequence of

Tau35, as well as a total tau antibody (Fig 1.13). Phosphorylation of tau at the epitopes corresponding to Ser202/Thr205, Thr231, Ser262/356 and Ser396/404 was detected by antibodies AT8, AT180, 12E8 and PHF1, respectively, and the amount of phosphorylated tau was standardised to total tau (Fig 4.5). In general, full-length tau showed a lower reactivity than Tau35 with AT8, 12E8 and PHF1, implying a relatively lower amount of phosphorylation of these epitopes in full-length tau, compared to Tau35. AT180 phosphorylation was equivalent in both CHO-FL and CHO-Tau35 cells. Interestingly, the different phospho-tau antibodies used to label CHO-Tau35 exhibited variable band patterns on blots, suggesting that Tau35 may be phosphorylated heterogeneously. The total tau antibody labelled an array of bands with three dominant Tau35 species at 39, 35, and 30 kDa. The 39 and 35 kDa species were picked up by all four phospho-dependent tau antibodies, indicating these two species undergo phosphorylation at multiple residues. The 30 kDa Tau35 species was reactive with AT8, AT180 and 12E8, but not with PHF1, possibly due to the absence of the C-terminal residues caused by secondary cleavage. Several Tau35 bands between 39 kDa and 35 kDa were labelled by AT8 and PHF1 exclusively, consistent with previous reports suggesting an association between AT8 and PHF1 reactivity and tau abnormality (Avila et al., 2006). Quantification of these results showed that, compared to full-length tau, the relative phosphorylation of Tau35 was increased by 170%, 200%, 70% and 220% at the AT8, AT180, 12E8 and PHF1 epitopes, respectively. Taken together, these results show that different Tau35 subpopulations are subjected to varying extents of phosphorylation and that the degree of Tau35 phosphorylation is significantly greater than that of full-length tau expressed in CHO cells.

Figure 4.5 Tau35 exhibits increased phosphorylation compared to full-length tau expressed in CHO cells



(A) Western blots of CHO-FL and CHO-Tau35 cells probed with antibodies against total tau and phosphorylated Tau (AT8: pSer202/pThr205; AT180: pThr231; 12E8: pSer262/pSer356 and PHF1: pSer396/pSer404). Molecular weight markers (kDa) are shown on the left. (B) Graphs show the ratio of phosphorylated tau to total tau in each cell type, relative to CHO-FL values, set to 100%. Values represent mean \pm S.E.M; n=3, one-way ANOVA, *P<0.05; **P<0.01.

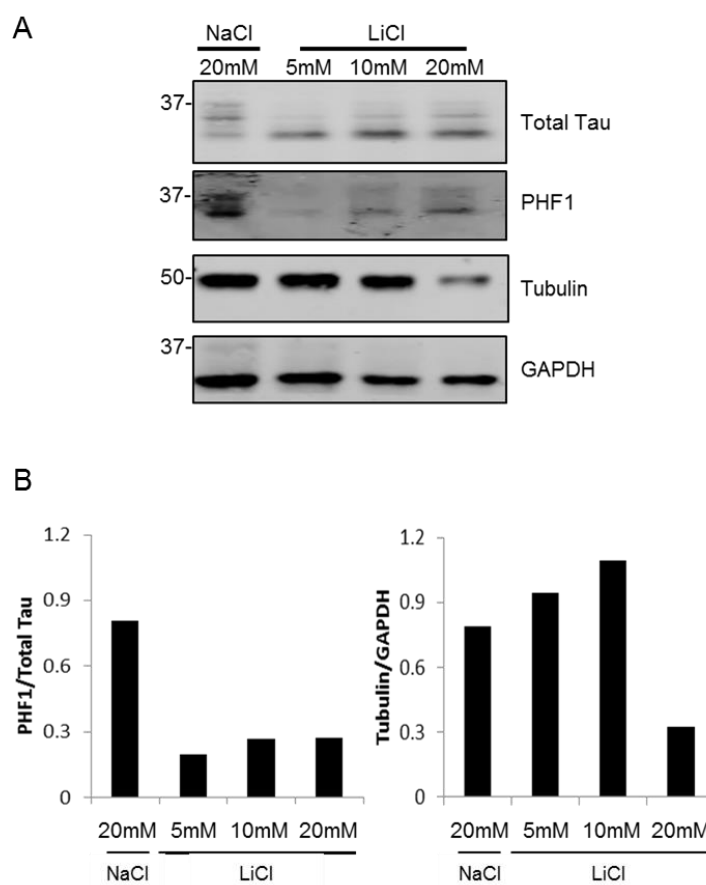
4.2.6 Down-regulation of tau phosphorylation is unable to enhance the microtubule binding ability of Tau35

The previous results showed that Tau35 is highly phosphorylated at Ser262/356 and Ser396/404, which are involved in regulating the binding of tau to microtubules. In order to verify whether increased phosphorylation is responsible for the lack of Tau35 binding to microtubules, cells were treated with LiCl, which reduces tau phosphorylation through suppressing GSK3 β activity, to determine whether the microtubule binding ability of Tau35 could be restored.

A pilot experiment was conducted to test the potency and the toxicity of different concentrations of LiCl on intracellular tau phosphorylation. CHO-Tau35 cells were treated with 5 mM, 10 mM, and 20 mM LiCl for 24 h using 20 mM NaCl as control. The amounts of tau phosphorylation and α -tubulin were determined on western blots (Fig.4.6). Each of the three concentrations of LiCl was sufficient to reduce tau phosphorylation at Ser396/404, with similar effectivity. However, 20 mM LiCl greatly decreased the protein level of α -tubulin. Based on this pilot experiment, 5mM LiCl was selected for further experiments, with 5mM NaCl as a control.

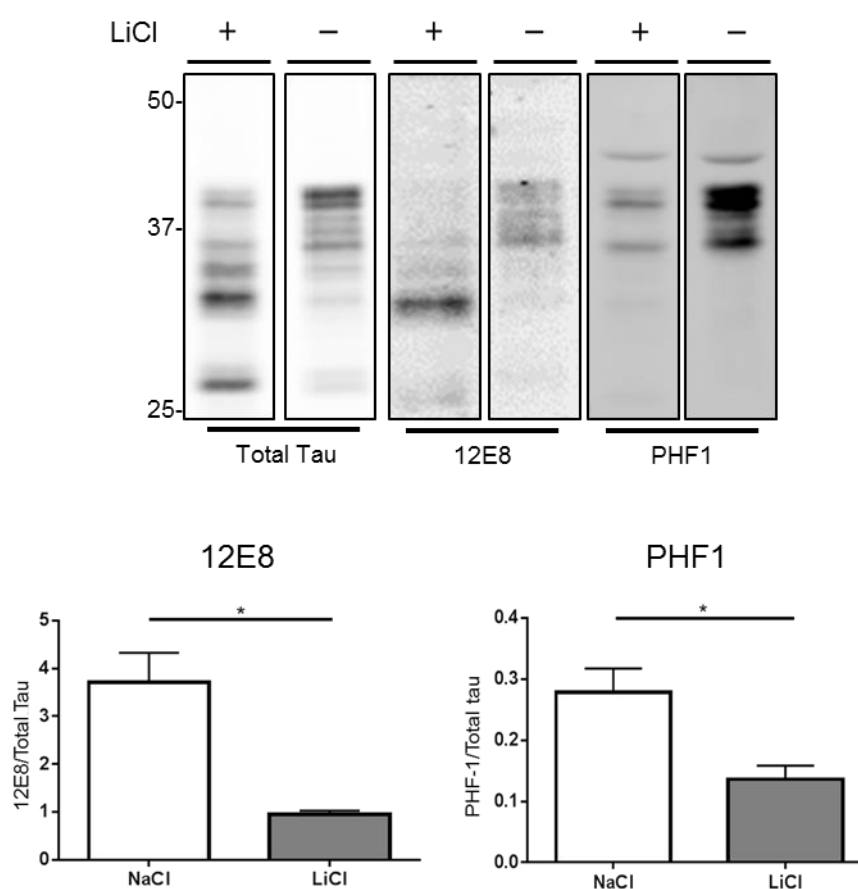
Western blots showed that compared to NaCl treated cells, LiCl treatment decreased phosphorylation of Tau35 by 70% and at Ser262/356 and 50% at Ser396/404, respectively, suggesting that GSK3 β participates in phosphorylation of Tau35 at these four residues (Fig.4.7). Notably, LiCl also affected the band pattern of Tau35, enriching smaller Tau35 species and reducing the amount of larger Tau35 species. These results suggest that the higher apparent molecular weight of some Tau35 species is due to phosphorylation. Interestingly, there was a significant decrease in the amount of Tau35 species of ~35 kDa, which is in keeping with previous findings of increased phosphorylation of the Tau35 isolated from the brains of patients with tauopathies.

Figure 4.6 Effect of LiCl on Tau35 phosphorylation in CHO cells



Western blots of CHO-Tau35 cells probed with antibodies against total tau, phosphorylated tau (PHF1: pSer396/pSer404), α -tubulin and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Molecular weight markers (kDa) are shown on the left. Graphs show the ratio of phosphorylated tau to total tau (bottom left) and the ratio of tubulin to GAPDH (bottom right). The results of this pilot experiment were obtained from one experiment.

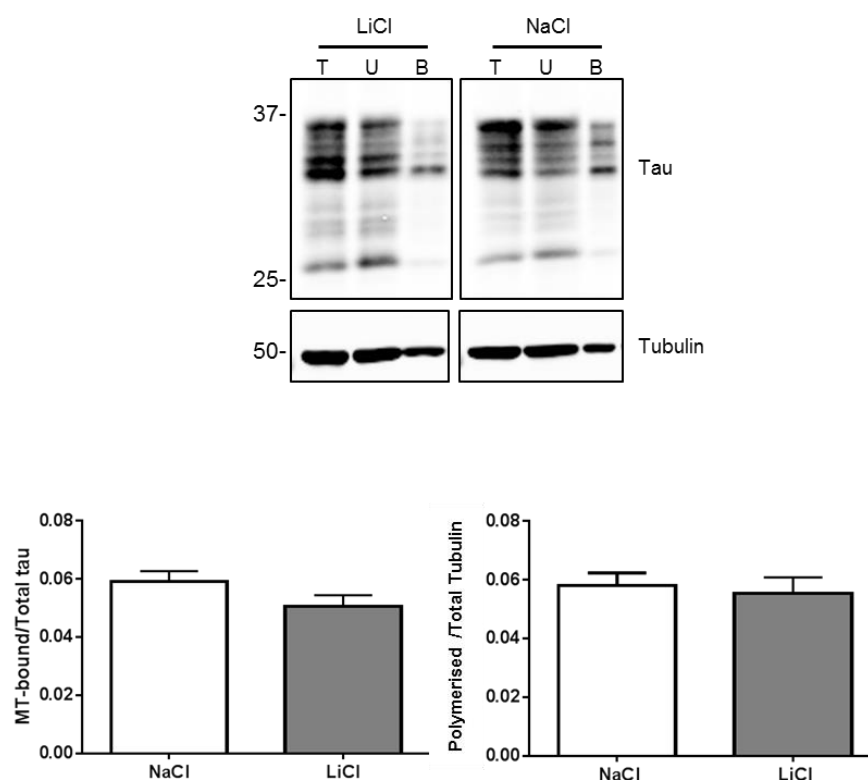
Figure 4.7 LiCl reduces Tau35 phosphorylation



Western blots of CHO-Tau35 cells treated with 5 mM LiCl (+) or 5 mM NaCl (–) with antibodies against total tau, and phosphorylated tau (12E8: pSer252/pSer356 and PHF1: pSer396/pSer404). Molecular weight markers (kDa) are shown on the left. Graphs show the ratio of phosphorylated tau to total tau. Values represent mean \pm S.E.M; n=3, Student t-test, *P< 0.05.

In situ microtubule binding assay shows that neither the proportion of microtubule-bound Tau35, nor the ratio of polymerised α -tubulin to total α -tubulin was increased by LiCl (Fig.4.8). These data show that blocking phosphorylation of Tau35 by inhibiting GSK3 does not restore the interaction between Tau35 and microtubules, indicating that the GSK3 β -mediated tau phosphorylation is unlikely the causative factor of the decreased interaction between Tau35 and microtubules.

Figure 4.8 LiCl is unable to enhance the interaction of Tau35 with microtubules



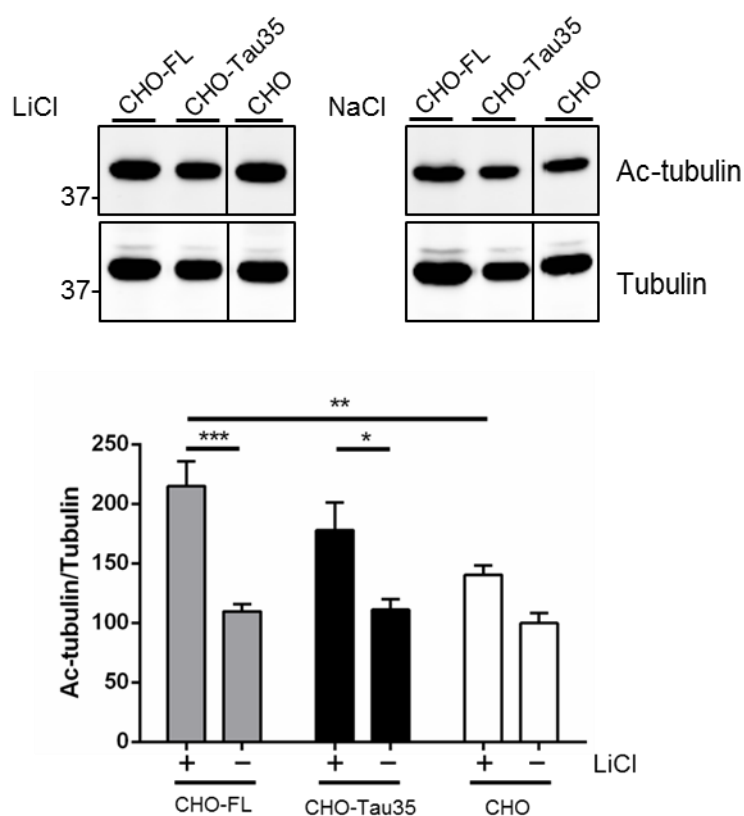
Western blots of total lysate (T), microtubule-unbound (U) and microtubule-bound (B) fractions of CHO-Tau35 treated with 5 mM LiCl or 5 mM NaCl for 24 h, probed with antibodies against total tau and α -tubulin (Tubulin). Molecular weight markers (kDa) are shown on the left. Graphs show the ratio of tau present in the microtubule-bound fraction to the total lysate (bottom left panel) and the ratio of polymerised α -tubulin to total α -tubulin (bottom right panel). Values represent mean \pm S.E.M; n=3.

4.2.7 GSK3 is involved in the regulation of tubulin acetylation

In addition to decreased phosphorylation level of tau, α -tubulin acetylation level is also affected by LiCl treatment. LiCl enhanced α -tubulin acetylation across all the three cell lines (Fig 4.9). Compared to NaCl treated group, the level of acetylated tubulin in CHO-FL cells increased by 100% and by 60% in CHO-Tau35 cells ($P < 0.05$). In contrast to CHO-FL and CHO-Tau35 cells, CHO cells also exhibited increased level of tubulin acetylation, but the difference is not statistically significant ($P > 0.05$). Moreover, within the LiCl treated group, the overall tubulin acetylation

level is 50% higher in CHO-FL cells compared to CHO ($P<0.05$), as well as a 26% increase in CHO-Tau35 versus CHO cells ($P>0.05$). This result suggests that GSK3 is also involved in the regulation of tubulin acetylation.

Figure 4.9 GSK3 is involved in the regulation of tubulin acetylation



Western blots of total lysates of CHO-FL, CHO-Tau35 and untransfected CHO cells probed with antibodies against acetylated α -tubulin (Ac-tubulin) and total α -tubulin (Tubulin). Cells were treated with 5 mM LiCl or 5mM NaCl for 24 h. Graph shows the ratio of acetylated α -tubulin to total α -tubulin relative to untreated CHO cells in cells treated with LiCl (+) or NaCl (-). Values represent mean \pm S.E.M, $n=3$, two-way ANOVA, * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Molecular weight markers (kDa) are shown on the left of the blot.

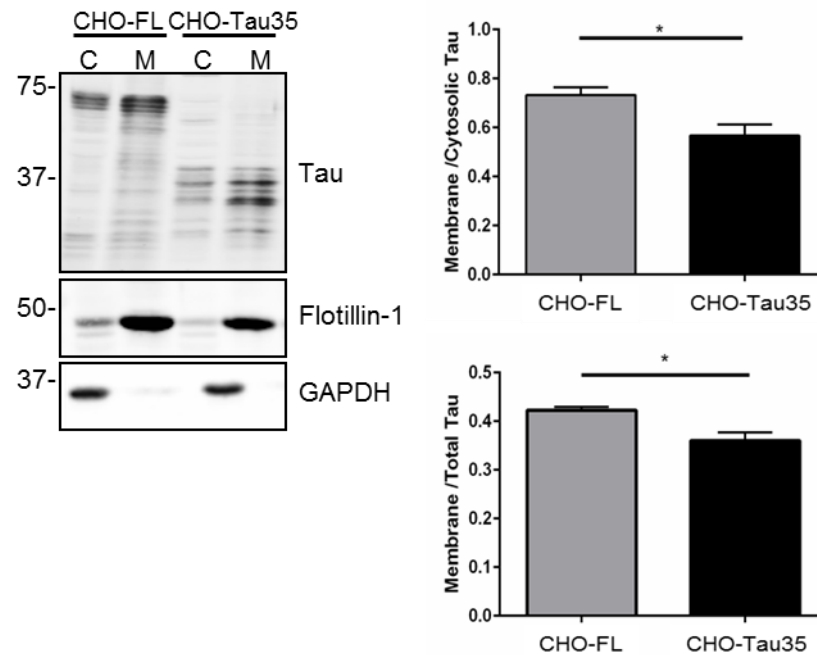
4.2.8 Tau35 is less prone to associate with plasma membrane than full-length tau

A growing number of studies have heightened the importance of tau in regulating signalling cascades. The execution of this role is partially based on the association of tau with the plasma membrane (Pooler and Hanger, 2010). Although the mechanism underlying tau-membrane association is unknown, it has been reported that the projection domain is involved in regulating the interaction, particularly in localising tau to the cell surface. However, studies have also suggested a role for the C-terminal half of tau in membrane binding (Brandt et al., 1995; Georgieva et al., 2014; Kunze et al., 2012). On the other hand, trafficking of tau between the cytosol and the neuronal membrane is dynamically regulated by tau phosphorylation, wherein plasma membrane-associated tau is primarily in a dephosphorylated state (Pooler et al., 2012).

In order to investigate the impact of the deletion of the N-terminal half of tau on the association between Tau35 and the plasma membrane, CHO-FL and CHO-Tau35 cells were fractionated into cytosolic (C) and membrane (M) fractions (Pooler et al., 2012). The distribution of tau between the cytosol and membrane was analysed on western blots probed with an antibody against total tau. For each cell line, 1% (v/v) of the total cytosolic fraction and 5% (v/v) of the membrane fraction was loaded, the value of each lane was adjusted according to the loading volume. The total amount of tau was determined by combining the values of the cytosolic and membrane fractions.

To confirm the stringency of the fractionation, cytosolic and membrane preparations were probed plasma membrane-associated flotillin-1 and cytosolic Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). As shown in Figure 4.9, Flotillin-1 was primarily localised in the membrane fraction, whereas α -tubulin and GAPDH were mostly localised in the cytosol. In CHO-FL cells, 42% of total tau was membrane-associated, whereas this proportion decreased to 36% in CHO-Tau35 cells, a reduction of 14%. Correspondingly, the ratio of membrane-associated tau to cytosolic tau decreased from 0.73 (arbitrary units) in CHO-FL to 0.56 in CHO-Tau35 cells, a decrease of 28%. These data show that deletion of the N-terminal half of tau negatively affects its association with the plasma membrane in CHO cells, although this association is not abolished, suggesting that the C-terminal half of tau also has a role in its binding to membranes.

Figure 4.9 Association of tau and Tau35 with the membrane in CHO cells



Western blots of cytosolic (C) and membrane fractions (M) of CHO-FL and CHO-Tau35 cells probed with antibodies against total tau, Flotillin-1, α -tubulin or GAPDH. Graph shows the ratio of tau present in the membrane fraction to cytosolic tau (upper panel) and to total tau (bottom panel). Values represent mean \pm S.E.M, n=3; Student t-test, *P<0.05.

4.3 Discussion

In this chapter, the effect of N-terminal cleavage of tau was investigated on its functions of microtubule binding and membrane-association in cells.

Firstly, expression of either full-length tau or Tau35 altered the shape of CHO cells, albeit in different ways. Compared to n CHO cells, full-length tau induced a more polygonal morphology, suggesting increased as stability of the cytoskeleton. In contrast, expression of Tau35 failed to recapitulate the effect observed with full-length tau, suggesting that Tau35 may have a decreased ability to stabilise microtubules.

Secondly, both the *in situ* microtubule binding assay and immunocytochemistry results confirmed that lack of its N-terminal half greatly decreases the ability of tau to

bind to microtubules, rendering Tau35 a more cytosolic protein. Moreover, full-length tau induced the formation of microtubule bundles, an effect that was not detectable with Tau35. Expression of Tau35 also caused a defect in tubulin acetylation, one of the prerequisites for the normal function of tubulin and a marker of stable microtubules.

Detachment of tau from microtubules is thought to be the initial step in the development and progression of tauopathies, and increased cytosolic Tau35 will be more liable to pathological changes including aberrant phosphorylation and aggregation. On the other hand, the disorganised microtubule structure, which is apparent when Tau35 is expressed in cells, may explain not only the changes in cell shape, but could also cause profound effects on other intracellular processes. Examples of such changes that could be affected by Tau35 expression include recruitment of other microtubule-associated proteins to microtubules, localisation of organelles, and transport of nutrients. In the context of neurons, integration of microtubules into the neuronal cytoskeleton is key for normal axonal structure and function, in which they act as tracks along which cargoes are transported to remote compartments, such as the synapse (Perdiz et al., 2011). Compromised microtubule integrity is regarded as being responsible for synapse loss, an early symptom of neurodegeneration (Scheff et al., 2006; Shankar and Walsh, 2009). The observed disability of tau in promoting tubulin acetylation is likely to further affect neuron polarisation, neurite branching and neurite outgrowth, posing threats to other compartments of neurons and neuronal viability (Yoshiyama et al., 2007).

In CHO-FL cells, MEC-17 expression enhanced microtubule bundling, but this effect was not apparent in CHO-Tau35 cells. Tau35 failed to cooperate with the tubulin acetyltransferase to regulate microtubule structure, further revealing the function of full-length tau, not only as a passive microtubule stabiliser, but also actively cooperating with other molecular processes involved in tubulin modification. Thus, Tau35 appears to disturb both the formation and the maintenance of proper microtubule structures in cells.

Thirdly, Tau35 is more prone to increased phosphorylation compared to full-length tau expressed in cells. Elevated tau phosphorylation may induce pathology through multiple mechanisms: (1) Increased phosphorylation of tau detaches tau from microtubules. In neurons, this detachment induces tau missorting from axons to the somatodendritic compartment, which can result in synaptic dysfunction (Zempel and Mandelkow, 2014). (2) Increasing phosphorylation of tau may alter its degradation

through the proteasome or by autophagic/lysosomal degradation, and its truncation by proteases (Wang et al., 2009). (3) Phosphorylation of tau may enhance tau aggregation (Avila et al., 2006). (4) Phosphorylation may change the association of tau with its interacting partners (Leugers et al., 2013; Morris et al., 2011; Pooler et al., 2012; Reynolds et al., 2008; Usardi et al., 2011). Overall, the increased phosphorylation of Tau35 relative to full-length tau may cause it to adopt pathological features in cells.

Following these findings, an important question is whether the decreased microtubule binding ability of Tau35 is permanent, due to the absence of structural components from the tau molecule, or reversible, due to decreased tubulin acetylation and increased tau phosphorylation. As shown in Figures 4.4 and 4.8, neither increasing tubulin acetylation, nor reducing cellular phosphorylation with LiCl, recovers the microtubule-binding ability of Tau35. These results illustrate the importance of the N-terminal half of tau in the tau-microtubule interaction and agreed with recent studies reporting N-terminally deletion of tau at Arg242 decreased interactions between microtubule and the resultant tau fragment (Matsumoto et al., 2015).

Finally, deletion of the N-terminal half of tau decreased, but did not eliminate, the association of Tau35 with membranes. This result demonstrates that Tau35, which lacks the projection domain of tau, only partially retains its ability to traffic into membranes, which support the findings that both the N-terminus and the MTB repeats of tau is able to interact with membrane (Brandt et al., 1995; Georgieva et al., 2014; Kunze et al., 2012). The localisation of tau to membranes confers functions of tau such as participation in intracellular signalling pathways and cell–cell signalling (Morris et al., 2011; Pooler and Hanger, 2010; Reynolds et al., 2008). Thus, reducing the amount of membrane-bound tau may directly disrupt functions related to this association. It is also possible that, although a significant proportion of Tau35 remains membrane-associated, the overall distribution pattern of tau is also disturbed. Such a change could be another route through which tau cleavage contributes to the aggravation of pathology because as well as being targeted to the plasma membrane, tau is also associated with intracellular membranes, including the endoplasmic reticulum and the Golgi apparatus (Brandt et al., 1995; Farah et al., 2005; Farah et al., 2006). However, a more detailed molecular analysis would be needed to fully elucidate how N-terminal cleavage of tau affects its membrane association.

In summary, in human tauopathies it is possible that N-terminal tau cleavage might lead to cell dysfunction in several different ways. Such dysfunction includes rendering tau into more toxic species, prohibiting tau from regulating cytoskeletal dynamics, and inhibiting important signalling functions of tau, perhaps through perturbing its association with membranes.

Chapter 5 UPR and Tau35 mediated degeneration

5.1 Introduction

Besides the loss-of-function effect resulting from tau truncation, another widely recognized consequence is the generation of tau fragments are prone to aggregate. Proteolytic tau fragmentation results in altered shape of tau molecule with potentially increased aggregation propensity (Von Bergen et al., 2005). Specifically, tau fragments containing the repeat domain have a higher tendency for aggregation, as proven by studies into tau fragments including Tau₁₅₁₋₃₉₁, Tau₁₋₄₂₁, Tau₂₄₃₋₄₄₁, Tau₁₋₃₆₈ (De Calignon et al., 2010; Matsumoto et al., 2015; Zhang et al., 2014b; Zilka et al., 2006). Truncation of tau in the N-terminal region has been increasingly reported (Amadoro et al., 2004; Corsetti et al., 2008; Derisbourg et al., 2015; Horowitz et al., 2004; Paholikova et al., 2015) but whether such truncations would promote tau aggregation remains to be proven.

In the previous chapter, phosphorylation of Tau35 was found to be increased at several different disease-related epitopes, compared to full-length tau. Under physiological conditions, tau phosphorylation is carefully maintained by networks of protein kinases and phosphatases. Amongst candidate tau kinases, altered expression and/or activity of GSK3 have been extensively reported to be associated with tau pathology and to be responsible for increased tau phosphorylation, which promote NFT formation (Hanger et al., 2009; Martin et al., 2011). Furthermore a possible link between decreased tubulin acetylation and GSK3 has also been suggested as inhibition of GSK3 is able to increase tubulin acetylation level (Nakakura et al., 2015). Therefore, the activity and expression of GSK3 α/β , as well as the upstream inhibitory protein kinase Akt, in CHO-FL and CHO-Tau35 cells was determined. Under basal conditions, GSK3 displays high constitutive activity, which can be regulated by phosphorylation. Phosphorylation at a conserved N-terminal serine residue (Ser21 in GSK3 α and Ser9 in GSK3 β) inhibits its kinase activity (Frame et al., 2001). It is widely accepted that Akt is the major mediator of this Ser9/21 phosphorylation and subsequent inactivation of GSK3 (Cross et al., 1995; Frame and Cohen, 2001). Thus, western blots using antibodies against phospho-Ser21 (GSK3 α) and/or phospho-Ser9 (GSK3 β) enabled analysis of the activity of GSK3 (Cole and Sutherland, 2008; Hur and Zhou, 2010; Sutherland et al., 1993). In contrast, phosphorylation of Akt at Ser473 is required for its full activation (Bellacosa et al., 1998). Hence, determining phosphorylation of Akt at Ser473 indicates the activation state of Akt (Liao and Hung, 2010).

Several lines of investigation have demonstrated a link between the unfolded protein response (UPR) and tau phosphorylation by GSK3 β (Cornejo and Hetz, 2013; Nijholt et al., 2013; Salminen et al., 2009). Activation of PERK, one of the master UPR sensors and has been observed in neurons and glia that exhibit tau pathology (Abisambra et al., 2013; Kohler et al., 2014), and PERK activity is upregulated during the early phase of AD and FTLT (Nijholt et al., 2012). *In vitro* studies have shown that induction of endoplasmic reticulum (ER) stress correlates with A β oligomer-induced tau phosphorylation, suggesting a link between ER stress, A β -mediated neurotoxicity and abnormal phosphorylation of tau (Resende et al., 2008). Similar findings were also obtained in several non-AD tauopathies, such as CBD, PSP, PiD and FTLT-tau. Increases in phospho-PERK (active PERK), phospho-eIF2 α and phospho-IRE1 in affected brain areas in these tauopathies have been reported by several groups (Nijholt et al., 2012; Stutzbach et al., 2013; Unterberger et al., 2006). Hoozemans and colleagues have also found that, in AD hippocampal neurons harbouring abnormally phosphorylated tau, phospho-PERK co-localises with GSK3 β , together with AT8 positive tau, suggesting that GSK3 β may link the UPR and tau pathology (Hoozemans et al., 2008). In contrast, activation of PERK-eIF2 α cascade facilitates the pathological phosphorylation of tau, and phosphorylated tau in rTg4510 mice, which express P301L tau, is reduced by treatment with a PERK inhibitor (Radford et al., 2015). *In vitro* studies have shown that activation of the UPR is correlated to increased activity of GSK3 β (Kim et al., 2005; Song et al., 2002) and this is probably due to the inhibition of Akt (Qin et al., 2010). Correspondingly, inhibiting GSK3 β by lithium chloride was able to protect tau from increased phosphorylation induced by thapsigargin, both *in vitro* and in rat brain (Fu et al., 2010). Taken together, these data suggest that UPR activation may lie upstream of GSK3 β activation and tau phosphorylation.

Another factor relevant to GSK3 activity is the role of insulin, with insulin resistance being a risk factor for certain tauopathies, leading to increased tau phosphorylation and NFT formation (El Khoury et al., 2014). Dysregulation of the Akt-GSK3 β pathway is regarded as both a cause and a consequence of insulin resistance. Following upstream stimulation by insulin, insulin receptor substrate-1 (IRS-1) was activated, which then phosphorylates PI3K. Phosphorylated PI3K subsequently facilitates Akt activation. Activated Akt mediates the phosphorylation of its multiple downstream targets involved in cellular growth and metabolism, including GSK3 β (Mackenzie and Elliott, 2014; Taha and Klip, 1999). Akt phosphorylates GSK3 β at

Ser9, and GSK3 α at Ser21, thereby abolishing the kinase activity of these GSK3 isoforms (Schinner et al., 2005).

Notably, increase GSK3 activity has been reported to be accompanied by UPR activation (Fu et al., 2010; Song et al., 2002). Possible mechanisms include the negative modulation of Akt or selective lysosomal degradation of inactive GSK3 (Nijholt et al., 2013; Qin et al., 2010). Therefore, experiments were performed to address the question of whether the UPR activation concurrently emerged with increased GSK3 activity in CHO-Tau35. Increased phosphorylation of PERK and eIF2 α is a widely reported marker of activation of the PERK branch of the UPR and is closely related to disease mechanisms in the tauopathies (Scheper and Hoozemans, 2013, 2015). Notably, phospho-eIF2 α is not a specific marker of UPR activation as it is the convergence point of the integrated stress response (ISR), wherein PERK is not the only eIF2 α kinases (Donnelly et al., 2013). Upregulation of CHOP, a pro-apoptotic executor downstream of PERK is also employed as an indicator of the occurrence of the detrimental outcome generated from UPR activation.

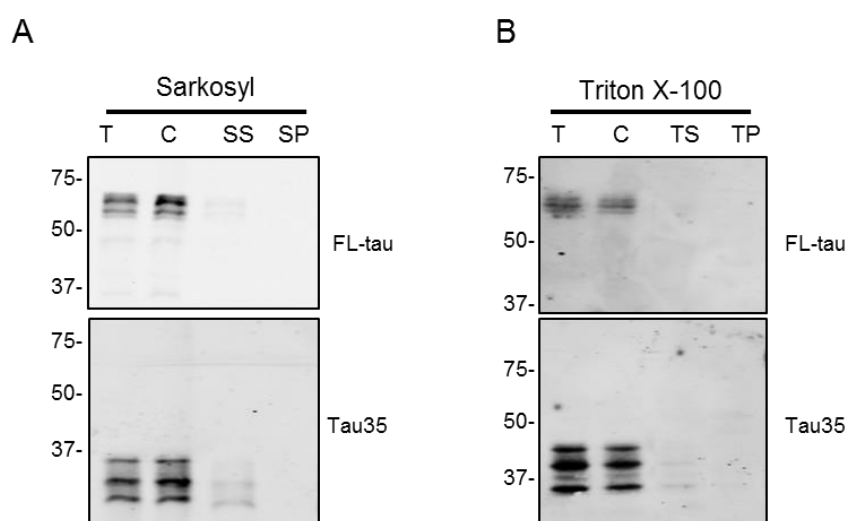
5.2 Results

5.2.1 N-terminally cleavage of tau does not induce formation of insoluble aggregates

Sarkosyl based fractionation has been widely used for isolation of insoluble tau aggregates, especially PHFs from AD related models (Forest et al., 2013; Greenberg and Davies, 1990; Schneider et al., 2004). On the other hand, the nonionic detergent Triton X-100 has also been used for isolation of insoluble tau aggregates from models of tauopathies besides AD (Guo et al., 2013; Guo and Lee, 2011; Waxman and Giasson, 2011; Xu et al., 2016). To investigate the effect of cleavage on the propensity of Tau35 to form aggregates, Sarkosyl/Triton X-100 insoluble fractionation assay was performed. Total cell lysates of CHO-FL and CHO-Tau35 were ultracentrifuge. Supernatants were taken as cytosolic fraction, and pellet was incubated with 1% Sarkosyl or 1% Triton X-100 and further separated into Sarkosyl/Triton X-100 soluble fraction and insoluble pellets. Western blotting analysis of these fractions has shown that for both full-length tau and Tau35, most of the protein were detected in cytosolic fractions, whereas minor amount of Tau35 was detected in the sarkosyl soluble-fraction (Fig 5.1A). However, no evident signal of tau was found in the Sarkosyl-insoluble fraction. Similar findings are obtained

from Triton X-100 fractionation assay (Fig 5.1A). This result suggests that although Tau35 adopted higher insolubility, it does not exhibit the propensity to form insoluble tau aggregates. It should be noted that given the limited number of experimental replicates, further experiments are still needed to confirm this observation.

Figure 5.1 N-terminally cleavage of tau does not induce formation of insoluble aggregates



(A) Western blots of total fraction (T), cytosolic fraction (C), Sarkosyl-soluble fraction (SS) and Sarkosyl-insoluble pellet (SP) of CHO-FL and CHO-Tau35 cells probed with antibody against total tau. (B) Western blots of total fraction (T), cytosolic fraction (C), Triton X-100 soluble fraction (TS) and Triton X-100 insoluble pellet (TP) of CHO-FL and CHO-Tau35 cells probed with antibody against total tau. Molecular weight markers (kDa) are shown on the left of the blots in A and B. Data acquired from one experiment.

5.2.2 The Akt-GSK3 pathway is less activated in CHO-Tau35

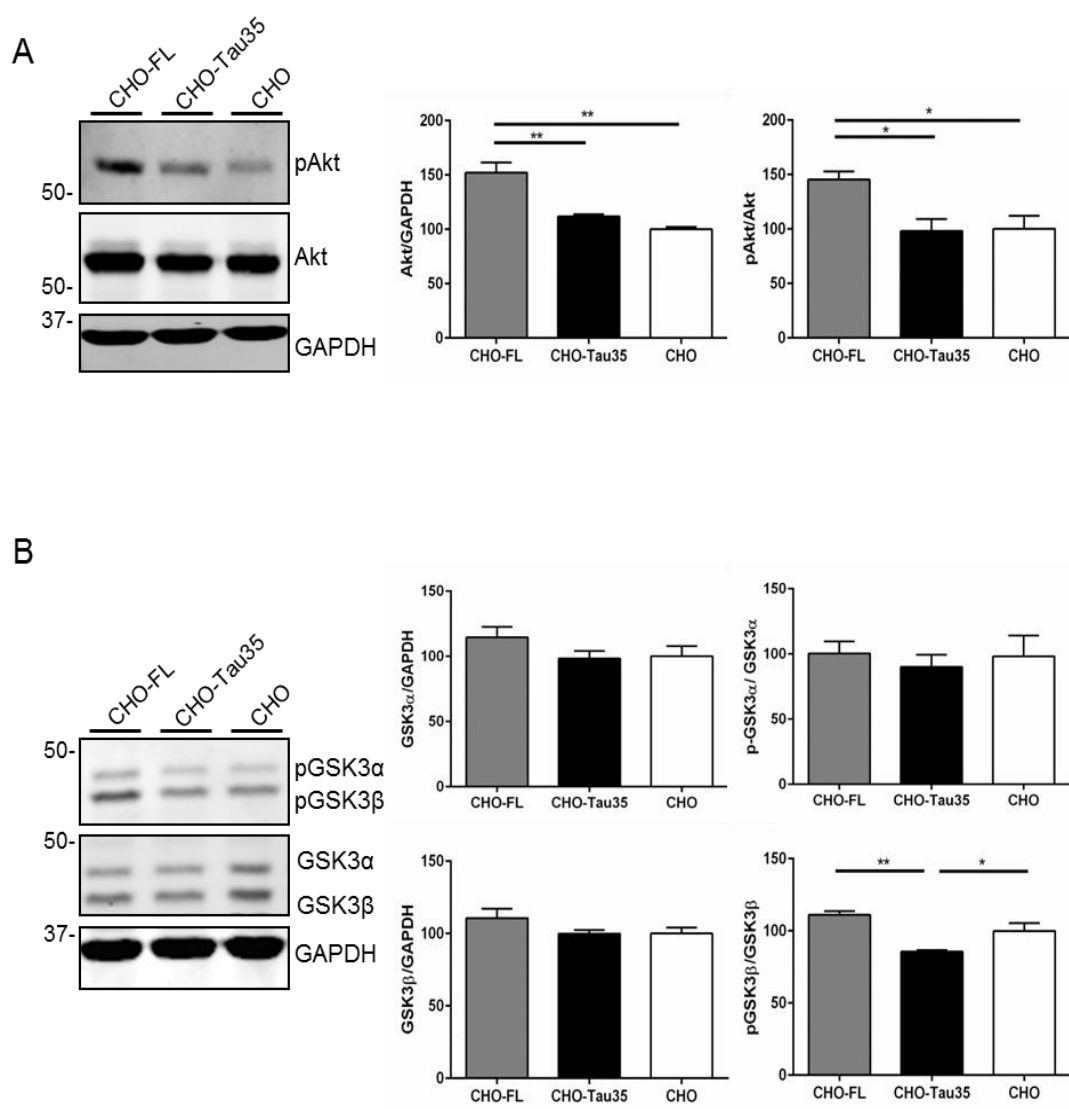
The amount and activity of Akt was assessed in CHO-FL, CHO-Tau35 and untransfected CHO cell lysates probed on western blots using antibodies against total Akt and phospho-Akt. The results showed that total Akt, relative to GAPDH, is increased by approximately 50% in CHO-FL cells, compared to both CHO-Tau35 and untransfected CHO cells (Fig. 5.1A). The ratio of phosphorylated Akt to total Akt

was also significantly elevated, indicating increased Akt activation, in CHO-FL cells. In contrast, total and active Akt were unchanged in Tau35 cells, compared to CHO cells. These results show that full-length tau causes enhanced Akt activity in CHO cells and this effect is not reproduced by Tau35, suggesting a novel role for the N-terminus of tau in Akt activation.

To assess GSK3 activity, antibodies recognising total and phospho-GSK3 (inhibitory phosphorylation at Ser21/Ser9 for GSK3 α/β) were used to probe the lysates from the three CHO cell lines on western blots (Fig. 5.1B). There was no difference in the total amount of GSK3 α or GSK3 β relative to GAPDH in any of the cell lines (Fig. 5.1B). However, there was a statistically significant decrease of 33% and 11%, respectively, in phospho/total GSK3 β in CHO-Tau35 cells, compared to CHO-FL and untransfected CHO cells, indicating increased GSK3 β activity induced by expression of Tau35 (Fig. 5.1B, lower panels). In contrast, GSK3 α inhibitory phosphorylation was unchanged by Tau35 expression in CHO cells (Fig. 5.1B, upper panels). These results suggest that Tau35, but not full-length tau, selectively increases GSK3 β activity in CHO cells, indicating a gain-of-toxicity caused by tau truncation.

Taken together, these results demonstrate that, unlike full-length tau, Tau35 inhibits the Akt/GSK3 β signal transduction pathway by decreasing Akt activity, which enables subsequent activation of GSK3 β , in the absence of any effect on GSK3 α activity.

Figure 5.2 The Akt-GSK3 pathway is less activated in CHO-Tau35



(A) Western blots of total lysates of CHO-FL, CHO-Tau35 and untransfected CHO cells probed with antibodies against (A) phospho-Akt, total Akt and GAPDH. Graphs show fold changes in relative to CHO cells in the ratios of total Akt to GAPDH (left panel) and phosphor-Akt to total Akt (right panel). (B) phospho-GSK3, total GSK3 and GAPDH. Graphs show fold changes in relative to CHO cells in the ratios of total GSK3α to GAPDH (upper left panel) and phospho-GSK3α to total GSK3α (upper right panel), total GSK3β to GAPDH (bottom left panel) and phospho-GSK3β to total GSK3β (bottom right panel). Molecular weight markers (kDa) are shown on the left of the blots in A and B. Values represent mean \pm S.E.M., n=4, one-way ANOVA, *P<0.05, **P<0.01.

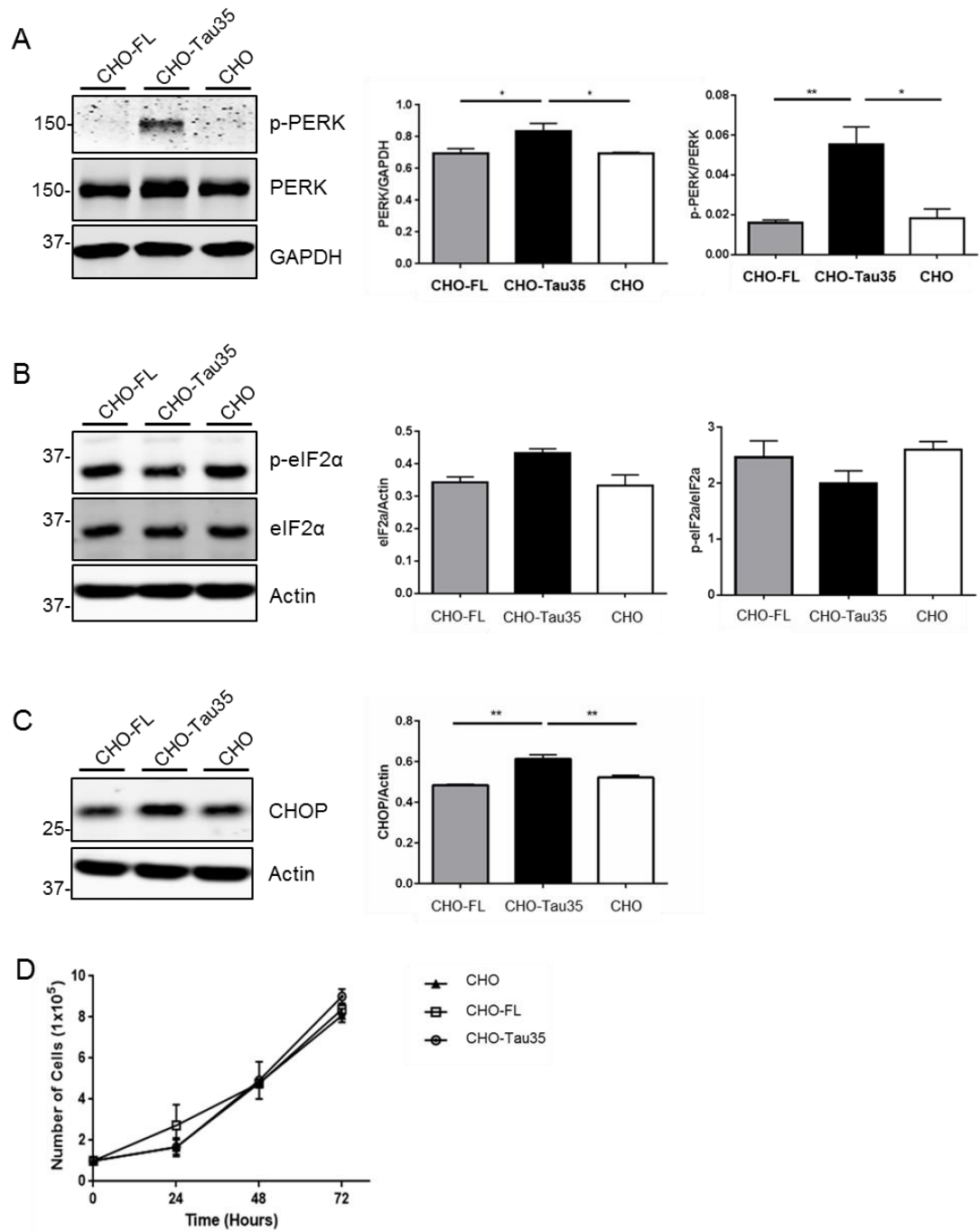
5.2.3 Expression of Tau35 in CHO cells activates the intrinsic UPR

In order to investigate the involvement of UPR activation in the dysregulation of Akt-GSK3 β pathway, the activation level of the PERK branch of the UPR pathway was assessed by determining the phosphorylation states of PERK and eIF2 α , and CHOP expression on western blots. As shown in Figure 5.2A, in the presence of Tau35, both expression (total PERK) and activation (phospho-PERK) of PERK are significantly increased in CHO-Tau35 cells compared to CHO-FL and CHO cells. The total amount of PERK was increased by 15% in CHO-Tau35 cells, and the ratio of phospho-PERK to total PERK was increased approximately 3-fold, in CHO-Tau35 cells compared to both CHO-FL and CHO cells. These findings show a substantial activation of PERK induced by Tau35 that is not apparent following expression of full-length tau and indicate activation by Tau35 of the PERK branch of the UPR in CHO cells.

The overall amount of active eIF2 α was unchanged in CHO-Tau35 cells compared to CHO-FL and CHO cells (Fig. 5.2B). This suggests that there may be a lag between activation of PERK and the downstream upregulation of eIF2 α , which could possibly be due to the PERK-independent regulating machinery of eIF2 α , as indicated by previous reports (DuRose et al., 2006; Osowski and Urano, 2011). In contrast to its expression in CHO-FL and CHO cells, expression of CHOP is elevated in CHO-Tau35 cells by approximately 26% and 17% ($P < 0.05$) respectively, whereas no significant change in the protein level of CHOP was found between CHO-FL and CHO cells (Fig. 5.2C). However, cell counting of each of the three CHO cell lines (Fig. 5.2D) indicated that there were no differences in the growth curves between the cell lines over a culture period of 72 hours. This finding shows that, despite upregulation of pro-apoptotic CHOP, cell viability is unaffected by Tau35 expression.

Taken together, these findings show that expression of Tau35 in CHO cells triggers activation of PERK, the upstream initiator of this branch of the UPR. Despite the modest change in eIF2 α activation, downstream upregulation of CHOP was induced by Tau 35 expression. However, these changes were not sufficient to affect cell proliferation. Increasing PERK activation and CHOP expression in CHO-Tau35 cells suggests that Tau35 expression could potentially sensitise cells against extracellular stimuli and stressors and/or cause other deleterious effects that are independent of cell death, such as translation repression or insulin resistance.

Figure 5.3 Expression of Tau35 in CHO cells activates the UPR



Western blots of total lysates of CHO-FL, CHO-Tau35 and CHO cells probed with antibodies against (A) phospho-PERK, total PERK and GAPDH. Graphs show the ratio of total PERK, over GAPDH (left) and activated PERK over total PERK (right), (B) phospho-eIF2α, total eIF2α and β-actin. Graphs show the ratio of total eIF2α over β-actin (left) and activated eIF2α, over total eIF2α (right), (C) CHOP and β-actin. The graph shows the ratio of CHOP over β-actin. A-C: molecular weight markers (kDa) are shown on the left of the blots. Values represent mean ± S.E.M, n=3, one-way ANOVA, *P<0.05, **P<0.01. (D) Cell growth curve of the three cell lines, n=4. Values represent mean ± S.E.M. (Cell counting experiment was performed by Miss Kanchan Halai)

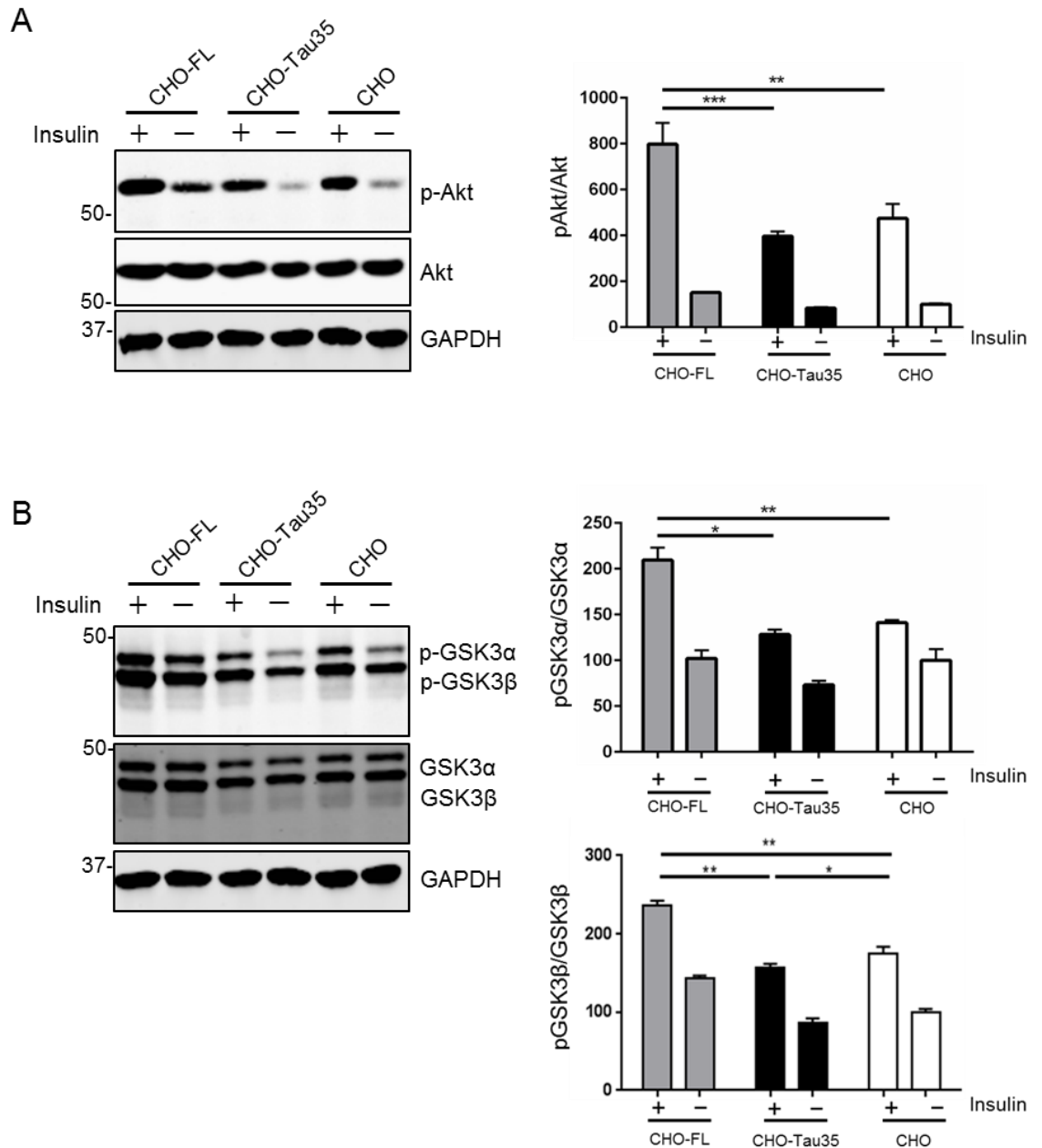
5.2.4 CHO-Tau35 is less sensitive to insulin induced Akt activation/GSK3 deactivation

Insulin regulates the Akt-GSK3 β pathway by activating IRS-1, which activates PI3K. Activated PI3K then phosphorylates lipids on the plasma membrane, producing second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Upon interaction with PIP3, Akt undergoes conformational changes, exposing residues Thr308 and Ser473 for phosphorylation by kinases, which induce full activation of Akt and leads to the inactivation of GSK3 (Hemmings and Restuccia, 2015; Manning and Cantley, 2007; Osaki et al., 2004). The role of Akt in the insulin-induced activation of glycogen synthase has previously been investigated in CHO cells (Takata et al., 1999). In order to determine whether expression of Tau35 and induction of the UPR affects the response of CHO cells to insulin, CHO-FL, CHO-Tau35 and CHO cells were treated with 100 nM insulin for 30 min. The responses of the three cell lines to insulin were assessed by measuring phosphorylation of Akt and GSK3 α/β .

Following exposure to insulin, Akt is activated by phosphorylation on residue Ser473, which then inactivates GSK3 α/β via phosphorylation on Ser21/9. As shown in Figure 5.4, exposure to insulin induced activation of Akt and inactivation of GSK3 α/β across all the three cell lines. Treatment with insulin led to a 4.4-fold increase in the amount of active Akt in CHO-FL cells and 3.7-fold increases in Akt activation in both CHO-Tau35 and CHO cells (Fig. 5.4A). Compared with insulin-treated CHO cells, the overall activation of Akt in CHO-FL cells was 68% higher, whereas it was 14% lower in CHO-Tau35 cells (Fig. 5.4A), indicating a potential loss of function induced by Tau35 expression.

Correspondingly, following exposure to insulin, Ser21 inhibitory phosphorylation of GSK3 α increased by 104% in CHO-FL cells, 75% in CHO-Tau35 cells, and 41% in CHO cells. Thus, in comparison to insulin-treated CHO cells, there was an overall increase of 48% in CHO-FL cells, and a 10% decrease in CHO-Tau35 cells, in GSK3 α inhibitory phosphorylation (Fig. 5.4B).

Figure 5.4 CHO-Tau35 is less sensitive to insulin induced Akt activation/GSK3 deactivation



Western blots of total lysates of CHO-FL, CHO-Tau35 and untransfected CHO cells. Cells were treated with (+) or without (-) 100 nM insulin for 30 min. (A) Cell lysates were probed with antibodies against phospho-Akt, total Akt and GAPDH. Graph shows fold changes in relative to untreated CHO cells in the ratio of phosphor-Akt to total Akt. (B) Cell lysates probed with antibodies against phospho-GSK3, total GSK3 and GAPDH. Molecular weight markers (kDa) are shown on the left of the blots in A and B. Graphs show fold changes in relative to untreated CHO cells in the ratio of phospho-GSK3α to total GSK3α (upper panel) and phospho-GSK3β to total GSK3β (lower panel). Values represent mean \pm S.E.M, n=6, two-way ANOVA, *P<0.05, **P<0.01, ***P<0.001.

Importantly, in comparison to untreated CHO cells, phosphorylation of GSK3 β at Ser9 increased by 64%, 80%, and 74% in insulin-treated CHO-FL, CHO-Tau35 and CHO cells, respectively, indicating a similar response in each cell line (Fig. 5.3B). Further, comparison of the insulin-treated groups showed that, compared to the CHO cells, inhibitory phosphorylation of GSK3 β increased by 35% in CHO-FL cells and decreased by 11% in CHO-Tau35 cells, indicating a higher activation of GSK3 β induced by Tau35 (Fig. 5.3B).

These results show that insulin-induced activation of Akt and GSK3 α/β inactivation is enhanced by expression of full-length tau in CHO cells. However, the effect of insulin on this signalling pathway in CHO-Tau35 cells is suppressed. These data indicate that expression of Tau35 may result in some symptoms of insulin resistance in CHO cells.

5.2.5 CHO-Tau35 cells are highly sensitive to extrinsically-induced activation of the UPR

To determine how the tau-expressing CHO cells respond to extrinsic UPR inducers, cells were exposed to dithiothreitol (DTT), or thapsigargin. DTT is a strong reducing agent, which blocks disulfide bond formation, rapidly leading to ER stress. Thapsigargin is a specific inhibitor of the sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase. Treatment with thapsigargin results in a decrease in ER calcium, thereby reducing the capability of calcium-dependent ER chaperones, and leading to accumulation of unfolded proteins. In order to induce acute ER stress, cells were treated with either 20 mM DTT for 2.5 h or 800 nM thapsigargin for 5 h. PERK, eIF2 α and IRE-1 were used as indicators of UPR activation under each condition.

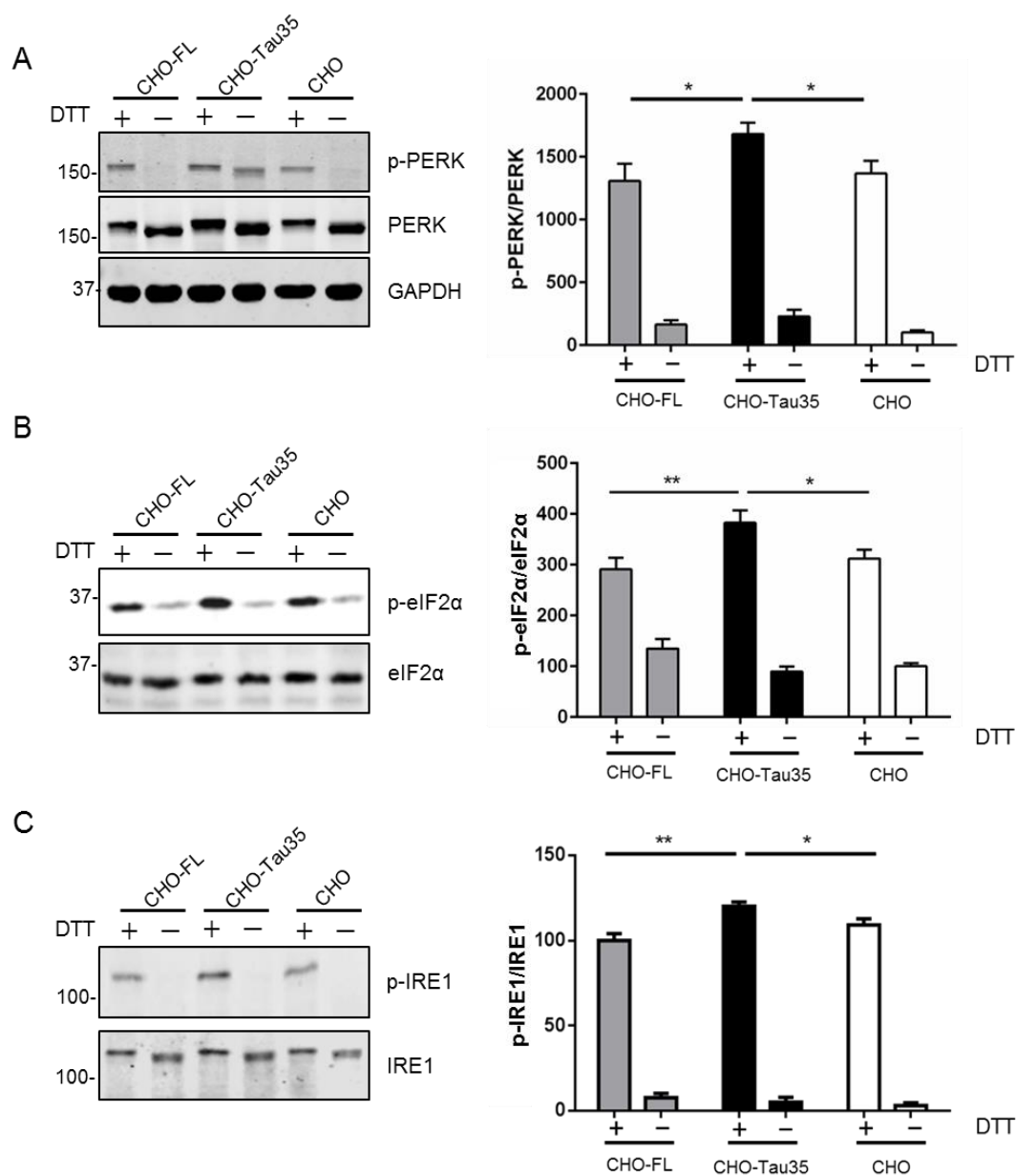
DTT caused a dramatic increase in the activity of all of the UPR markers, reflecting the induction of the UPR in the CHO cell lines (Fig. 5.4). Activation of PERK, eIF2 α and IRE1 in the CHO-Tau35 cells was significantly greater ($P < 0.05$) than in either CHO-FL or CHO cells (Fig. 5.4). The ratio of phospho/total PERK in CHO-Tau35 cells was increased by 28% and 23%, compared to DTT-treated CHO-FL and CHO cells, respectively (Fig. 5.4A). Correspondingly, eIF2 α activation was increased in DTT-treated CHO-Tau35 cells by 31%, and 22% compared to both CHO-FL and CHO cells respectively (Fig. 5.4B). These results indicate that the PERK branch of the UPR is highly activated by DTT treatment in CHO-Tau35 cells. In addition, phosphorylation of IRE1 in DTT-treated CHO-Tau35 cells was increased by 20%

and 10%, compared to CHO-FL and CHO cells, respectively, suggesting that the IRE1 branch of the UPR is activated to a higher degree in CHO-Tau35, compared to CHO-FL and untransfected CHO cells.

Thapsigargin treatment also activated the UPR, as assessed by phospho-PERK, phospho-eIF2 α and IRE1 (Fig. 5.5). After treatment with 800 nM thapsigargin for 5 h, CHO-Tau35 cells displayed a higher overall activation of both PERK and IRE1 compared with CHO-FL and CHO cells. (Fig. 5.5A, C) CHO-Tau35 cells displayed 14% and 10% increases in phospho/total PERK, compared to CHO-FL and CHO cells, respectively after exposure to thapsigargin. Elevated PERK activation in CHO-Tau35 cells led to increases of 26% and 12% more activated eIF2 α comparing with CHO cells and CHO-FL cells, respectively. Furthermore, IRE1 exhibited an 80% increase in activation in CHO cells expressing Tau35, compared to those expressing full-length tau and IRE1 was also increased by 45% compared to untransfected CHO cells.

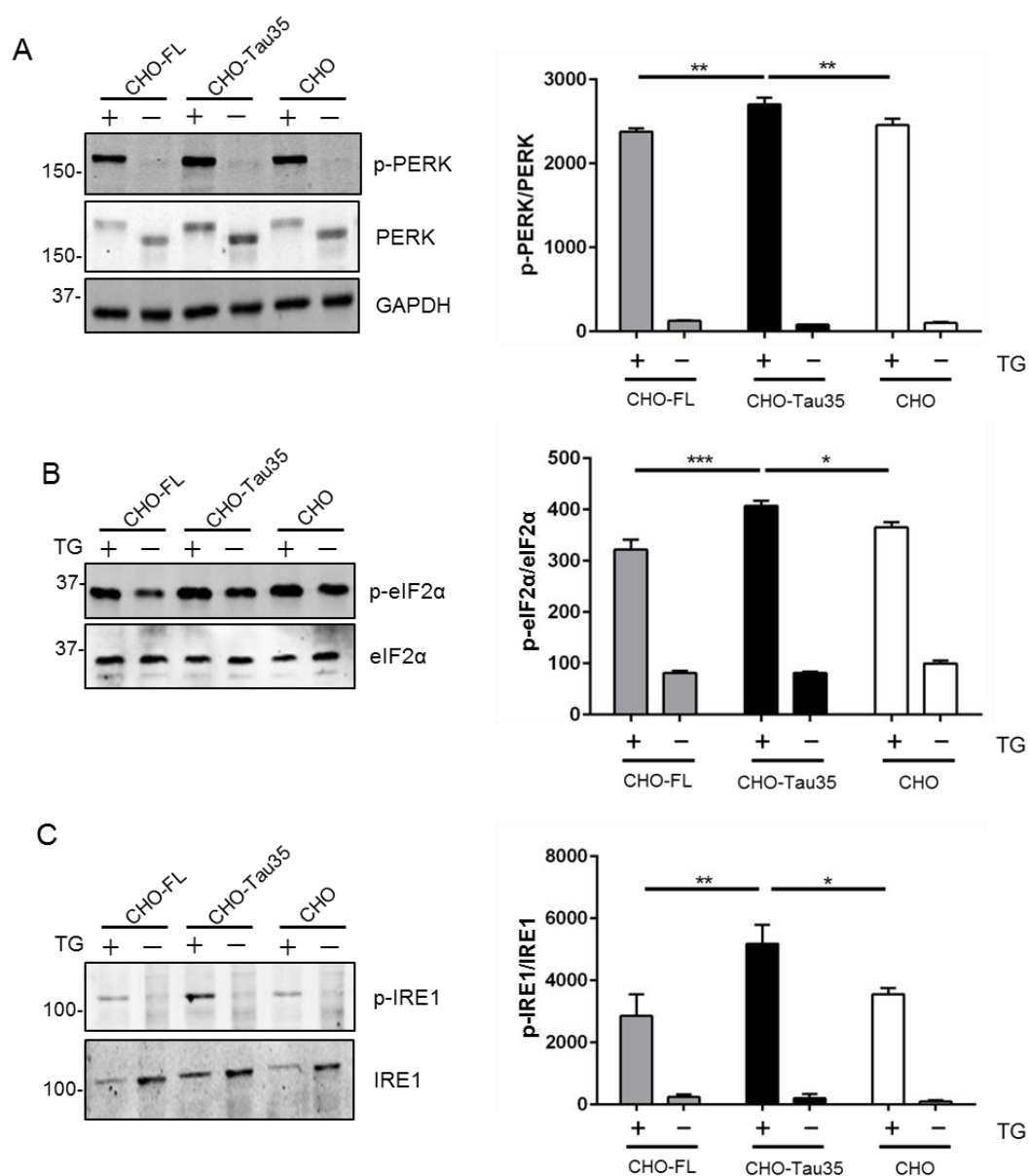
Taken together, the presence of Tau35 potentiates the effects of pharmacological induction of both the PERK branch and IRE1 branch of the UPR in CHO cells, indicating a potential gain of function of Tau35 in this cell model.

Figure 5.5 CHO-Tau35 cells are highly sensitive to DTT-induced activation of the UPR



Western blots of total lysates of CHO-FL, CHO-Tau35 and CHO cells. Before lysis, cells were treated with or without 20 mM DTT for 2.5 h. (A) Cell lysates probed with antibodies against phospho-PERK, total PERK and GAPDH. Graph shows fold changes in relative to untreated CHO cells in the ratio of phospho-PERK to total PERK. (B) Cell lysates probed with antibodies against phospho-eIF2α and total eIF2α. Graphs show fold changes in relative to untreated CHO cells in the ratio of phospho-eIF2α to total eIF2α. (C) Cell lysates probed with antibodies against phospho-IRE1 and total IRE1. A-C: Molecular weight markers (kDa) are shown on the left of the blots. Graphs show fold changes in relative to untreated CHO cells in the ratio of phosphor-IRE1 to total IRE1. Values represent mean \pm S.E.M, $n=4$, two-way ANOVA, * $P<0.05$, ** $P<0.01$.

Figure 5.6 CHO-Tau35 cells are highly sensitive to thapsigargin induced activation of the UPR



Western blots of total lysates of CHO-FL, CHO-Tau35 and CHO cells. Before lysis, cells were treated with or without 800 nM thapsigargin (TG) for 5 h. (A) Cell lysates probed with antibodies against phospho-PERK, total PERK and GAPDH. Graph shows fold changes in relative to untreated CHO cells in the ratio of phospho-PERK to total PERK. (B) Cell lysates probed with antibodies against phospho-eIF2α, and total eIF2α. A-C: Molecular weight markers (kDa) are shown on the left of the blots. Graphs show fold changes in relative to untreated CHO cells in the ratio of phospho-eIF2α to total eIF2α. (C) Cell lysates probed with antibodies against phospho-IRE1 and total IRE1. Graphs show fold changes in relative to untreated CHO cells in the ratio of phospho-IRE1 to total IRE1. Values represent mean \pm S.E.M., n=4-6, two-way ANOVA, *P<0.05, **P<0.01, ***P<0.001.

5.3 Discussion

In this chapter, the solubility of Tau35 expressed in CHO cells was assessed. Tau35 did not exhibit a strong propensity to form insoluble tau aggregates as revealed by both Sarkosyl and Triton X-100-based fractionation assays. In contrast to C-terminal truncation of tau, whether N-terminal truncation contributes to the aggregation of tau remains unclear. On the other hand, although intracellular tau deposits have been considered causative of neuronal death, more and more evidence has shown that other species of tau, such as soluble misfolded tau, abnormally phosphorylated tau (Takashima, 2008), and mislocalised tau (Gilley et al., 2016; Zempel and Mandelkow, 2014), are able to exacerbate cytotoxicity in the absence of overt or coinciding formation of such deposits (Kopeikina et al., 2012). Hence, it is reasonable to speculate soluble Tau35 is sufficient to cause deleterious effects to cells.

These results further show that the Akt-GSK3 β pathway is selectively dysregulated in CHO-Tau35 cells, resulting in reduced Akt activation and decreased inhibitory phosphorylation (i.e. increased activity) of GSK3 β , without significantly affecting GSK3 α activity. Furthermore, expression of Tau35 leads to the induction of the intrinsic UPR indicated by phosphorylation of PERK and increased expression of CHOP. Further experiments showed that insulin is less efficient in suppressing GSK3 β activity in CHO-Tau35 cells through the activation of Akt. Importantly, CHO-Tau35 cells exhibit enhanced sensitivity to both DTT and thapsigargin induced UPR activation.

Increased activation of GSK3 α/β not only explains the higher phosphorylation level of Tau35 compared to full-length tau in CHO-tau cells, but it may also contribute to the decreased tubulin acetylation observed (Chapter 4). Inhibiting GSK3 β with lithium chloride restored tubulin acetylation, most likely through reducing the activity of HDAC6, a tubulin deacetylase (Chen et al., 2010). Furthermore, activation of GSK3 is linked to cell death, particularly to apoptosis elicited by extrinsic inducers such as thapsigargin in SH-SY5Y cells (Song et al., 2002). Studies in mouse insulinoma cells have demonstrated that attenuation of the inhibitory phosphorylation of GSK3 β leads to ER stress-mediated apoptosis, which indicates another route through which dysregulated GSK3 β could contribute to human tauopathies (Srinivasan et al., 2005).

Accumulating evidence suggests that prolonged activation of the UPR is emerging as a contributor that underlies the progression several neurodegenerative diseases, including AD and related tauopathies (Ferreiro and Pereira, 2012). Over-activation of the UPR may be detrimental to cells through several different routes. It has been demonstrated that persistent UPR activation subsequently activates JNK, disrupting the insulin-PI3K-Akt-GSK3 pathway. CHOP is well recognised as a pro-apoptotic factor, thus increased CHOP expression may contribute to cell death via apoptosis (Tabas and Ron, 2011). However, the absence of any overt cell death of any of the three cells lines used here, regardless of chronic activation of PERK and increased CHOP may be attributed to the concomitant activation of potentially protective factors, such as sXBP1. sXBP1 is a transcription factor that directly targets genes that facilitate the refolding and degradation of misfolded proteins, including ER chaperones such as Grp78/BiP, Grp58, Grp94, ER-associated degradation (ERAD) components (Ron and Walter, 2007). Interestingly, the protective response of the UPR can also act as a driver of neuropathology. Activation of the PERK branch of the UPR decreases global protein synthesis to reduce the ER load. However, in the context of neurodegeneration, attenuation of global protein translation results in synaptic failure, neuronal loss and clinical disease in a mouse model of frontotemporal dementia (Radford et al., 2015). Therefore, it would be of interest to determine whether activation of PERK in CHO-Tau35 cells can also lead to attenuation of protein synthesis. Although not tested here, it is also possible that synaptic components could be targeted through a similar activation of the UPR in neurons.

Further experiments described herein showed that insulin is inefficient in triggering the activation of Akt and the downstream inactivation of GSK3 α/β . These findings further support the view that insulin resistance may be associated with the development of AD, proposing the diabetic condition as a potential risk factor for tau-related dementia (El Khoury et al., 2014). Indeed, reports suggest that there may be a correlation between diabetes and dementia (Verdile et al., 2015). Although activation of the UPR offers a plausible explanation for the dysregulation of the insulin-Akt-GSK3 β signal transduction pathway, other mechanisms should not be excluded. For example, full-length tau is able to bind to other partners, such as the p85 α subunit of PI3K, an upstream regulator of Akt (Reynolds et al., 2008). Thus, disruption of the interaction between tau and PI3K may also negatively impact on Akt activity. However, more molecular details are still needed for further elucidating the impact of the deletion of the N-terminal half of tau on this interaction.

CHO-Tau35 cells show increased sensitivity to extrinsic inducers of ER stress. Indeed, after pharmacological induction of acute ER stress, the activation of key components of the UPR, namely PERK, eIF2 α , and IRE1, are increased in CHO-Tau35 cells compared to CHO-FL and CHO cells. Inflammatory cytokines have been reported to be physiological extrinsic inducers of the UPR (Chaudhari et al., 2014). Expression of pro-inflammatory cytokines, such as Tumor necrosis factor α (TNF- α), interleukin 1 α and interleukin 1 β (IL-1 α and IL-1 β), is enhanced in the brains of people affected by AD, compared with age-matched controls (McGeer and McGeer, 2002; Sastre et al., 2008). A direct link was established between inflammation and tau phosphorylation in experiments showing that activation of microglia with lipopolysaccharides (LPS) or A β , induced neuronal tau phosphorylation when co-cultured with rat primary neocortical neurons (Arnaud et al., 2006). Therefore, assessing the response of CHO-Tau35 cells to cytokine-induced UPR activation may provide new clues for understanding the role of neuroinflammation that underlies tau-mediated neurodegeneration.

An interesting question is what causes activation of the UPR in CHO-Tau35 cells. In the context of neurodegeneration, accumulation of abnormal or misfolded proteins has been proposed as a primary trigger of the UPR (Ho et al., 2012). Supporting this notion, tau species with high apparent molecular weight on western blots of CHO-Tau35 cells have been detected (Chapter 3). This result, combined with the absence of insoluble Tau35 deposits (Fig 5.1), suggests soluble Tau35 oligomers as the toxic species in CHO-Tau35 cells. In parallel, a study using primary neurons revealed that ER stress and phosphorylation of tau can induce each other, suggesting that soluble, highly phosphorylated tau *per se* is sufficient to facilitate the activation of the UPR (Ho et al., 2012).

Taken together, expression of Tau35 in CHO cells caused over activation of two highly inter-related signalling pathways, namely insulin-Akt-GSK3 β and the UPR pathway. Dysregulation of these two pathways could lead to further tau abnormalities and cell dysfunction, which suggests possible mechanisms through which tau fragmentation could result in the development of disease and human tauopathies.

Evidence has shown that increased activation of GSK3 β is closely correlated with tau-mediated neurodegeneration through the generation of highly phosphorylated tau (Hanger and Noble, 2011). In addition, recent studies have also shown that

inhibition of GSK3 β upregulates tubulin acetylation by attenuating the activity of the tubulin deacetylase, HDAC6 (Chen et al., 2010), which could provide a possible explanation for the altered tubulin acetylation detected in CHO-Tau35 cells (Chapter 4). In CHO-Tau35 cells, lower Akt activation level was observed, resulting in an upregulation of GSK3 activity, as shown by decreased phosphorylation at Ser21/9. These observations, combined with accumulating evidence proposing GSK3 β as a critical link to multiple pathogenic cascades. These findings also raise important questions as to (1) the cause of the increased phosphorylation of Tau35, (2) the impact of this dysregulation on the cell physiology, and (3) how these upstream abnormalities contribute to the further development of pathological changes relevant to the development of tauopathy in humans.

Chapter 6 Discussion

In a CHO cell model stably expressing a tau fragment associated with human tauopathy (Tau35), N-terminal cleavage of tau dramatically decreased its microtubule binding ability, leading to microtubule instability and defective microtubule bundling. Phosphorylation of Tau35 is significantly higher than that of full-length tau expressed in CHO cells. Furthermore, Tau35 elicited activation of the unfolded protein response (UPR), dysregulated the insulin-Akt-GSK3 β pathway, and increased vulnerability to extrinsic stressors. These data indicate that pathological truncation of tau not only results in the loss-of-function of tau, but also exerts a gain-of-toxicity effect by disrupting critical intracellular pathways, which further facilitate pathological changes in tau.

6.1 A cell model of Tau35-mediated degeneration

Previously, this laboratory described a 35 kDa C-terminal tau fragment (Tau35), lacking the N-terminus of tau but containing four microtubule-binding repeats (4R), that was readily detectable in PSP, CBD degeneration and 4R tau-related forms of FTLD, but was absent from healthy controls (Wray et al., 2008). Further studies using a Tau35-expressing mouse model revealed that minimal expression of this tau fragment was sufficient to recapitulate key features of human tauopathies, including progressive cognitive and motor deficits, autophagic/lysosomal dysfunction, loss of synaptic protein, and reduced life-span. Moreover, sodium 4-phenylbutyrate was able to reverse the observed abnormalities in Tau35 mice (Bondulich et al., 2016).

To verify the observed loss of tau function in Tau35 mice, the ability of Tau35 to bind to and stabilise microtubules, which are fundamental tau functions, was tested in a CHO cell model stably expressing Tau35. These experiments showed that both the microtubule binding ability of Tau35 and tubulin acetylation in CHO-Tau35 cells were both significantly decreased, compared to CHO-FL cells. In contrast to CHO-FL cells, no evident microtubule bundles are visible in CHO-Tau35 cells, indicating compromised stability of microtubule structure which cannot be restored by MEC-17, an acetyltransferase reported to rescue axonal transport deficits caused by leucine-rich repeat kinase 2 (LRRK2) mutations (Godena et al., 2014). These results indicate that Tau35 not only lacked the function of tau as a microtubule stabiliser, but also disrupted the maintenance of microtubule structure. Correspondingly, after promotion of tubulin acetylation using MEC-17 over expression in CHO cells, Tau35

failed to facilitate microtubule bundling as effectively as full-length tau, further supporting the loss-of-function of Tau35.

Notably, increased phosphorylation of Tau35 was observed in CHO-Tau35 cells, in line with the established elevation of tau phosphorylation in a range of tauopathies (Hanger et al., 2009; Wray et al., 2008). It is known that phosphorylation at Ser262, Thr231 and Ser235 on tau reduces its binding to microtubules (Sengupta et al., 1998). Therefore, to dissect the role of the C-terminal half of tau in relation to tau phosphorylation, inhibition of GSK3 β using LiCl was used to reduce tau phosphorylation in CHO-Tau35 cells. Although LiCl successfully reduced phosphorylation of Tau35, no restoration of the microtubule binding ability of Tau35 was detected after LiCl treatment. This result suggests that the inability of Tau35 to bind to microtubules is correlated with the lack of the N-terminus of tau, highlighting the role of the N-terminal half of tau in mediating tau-microtubule interaction. These data thus add significantly to understanding of the functions of the N-terminal half of tau, which has become of increasing interest in the field (Amadoro et al., 2004; Derisbourg et al., 2015; Matsumoto et al., 2015; Paholikova et al., 2015).

Tau35 also displayed a reduced affinity to associate with membranes. This finding is in line with the findings indicating that both the N-terminus and the microtubule binding domain are able to interact with membrane (Brandt et al., 1995; Georgieva et al., 2014; Kunze et al., 2012). The binding of tau to the plasma membrane and to lipid-rich membranes is correlated with several functions of tau, such as its participation in intracellular signalling pathways (Lee et al., 2005; Reynolds et al., 2008; Wang and Liu, 2008), serratation (Perez et al., 2016; Pooler et al., 2013), its involvement in cell-cell signalling (Gomez-Ramos et al., 2009; Gomez-Ramos et al., 2008), and regulating neuronal development (Brandt et al., 1995; Kempf et al., 1996). Thus, altered interaction patterns between Tau35 and membranes may also affect such processes and contribute the progression of disease. However, further details are still needed to fully elaborate these processes under both physiological and pathological conditions.

This study also showed that the activity of GSK3 β , a major tau kinase, is upregulated in CHO-Tau35 cells, whereas the activity of GSK3 α was unchanged. The amount and activity of Akt, responsible for the upstream inhibitory phosphorylation of GSK3 β , were both decreased in CHO-Tau35 cells, compared to CHO-FL cells, which may provide a partial explanation for the observed increase in

GSK3 activity (Bouzakri et al., 2005; Correia et al., 2012; El Khoury et al., 2014). Interestingly, symptoms of insulin resistance were also observed in CHO-Tau35 cells because the insulin-Akt-GSK3 β pathway was less responsive to insulin, when compared to both CHO-FL and CHO cells.

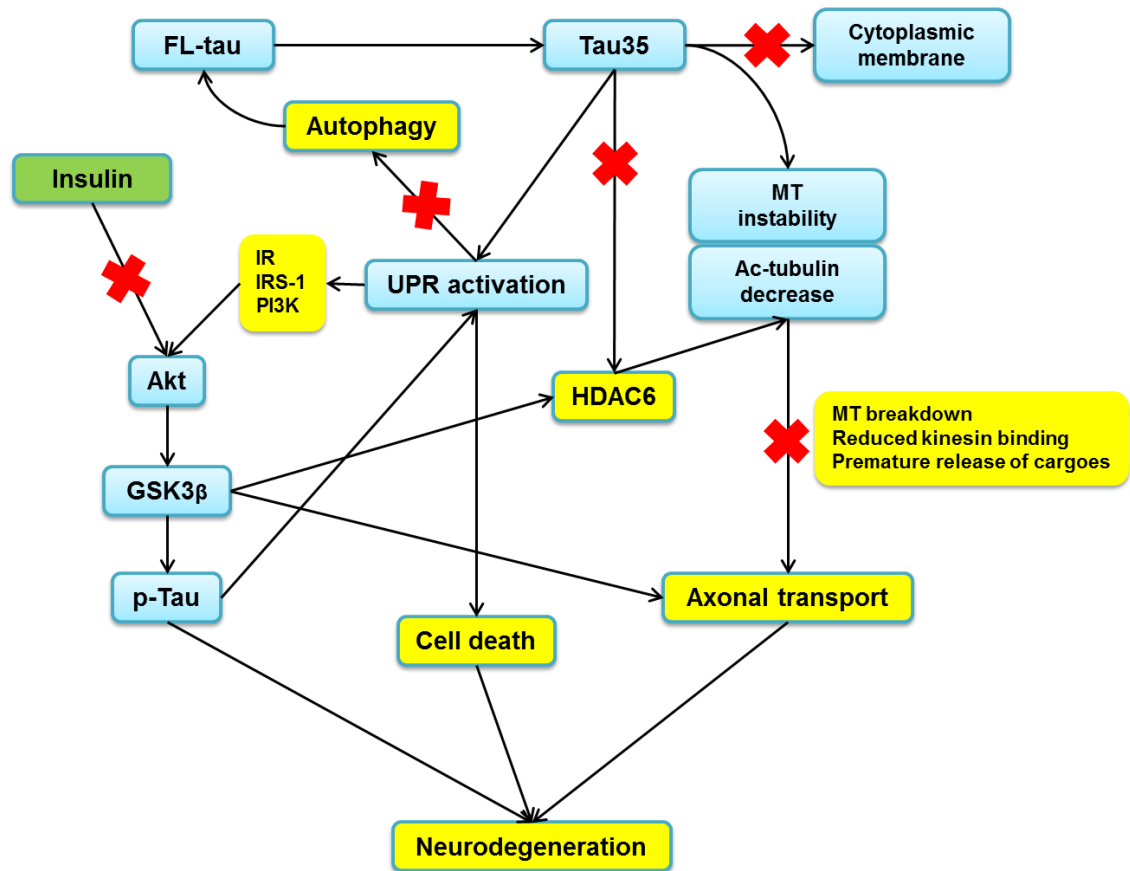
It is reported that activation of the UPR negatively regulates Akt (Qin et al., 2010). Thus, to investigate the role of UPR in the dysregulated Akt-GSK3 β pathway, the status of the UPR was investigated, both intrinsically and following extrinsic UPR induction using DTT or thapsigargin. CHO-Tau35 cells exhibited initiation of the intrinsic UPR as reflected by the activation of PERK, a key component of the PERK branch of the UPR, as well as CHOP, a pro-apoptotic executioner of the UPR (Figure 1.10). Moreover, the UPR was activated to a greater extent in CHO-Tau35 cells compared to both CHO-FL cells and untransfected CHO cells under the same conditions of DTT and thapsigargin treatment. Taken together, these results suggest that Tau35 can exert toxic effects via disruption of both the Akt-GSK3 β and UPR pathways in CHO cells. Meanwhile the tau fragment sensitised CHO cells to extrinsic UPR inducers, making Tau35-expressing cells more vulnerable to insults. A growing body of evidence has shown a correlation between the UPR and the development of tauopathy, including in AD and FTLT-tau (Pereira, 2013). Recent reports demonstrate that prolonged activation of the UPR resulted in attenuated synthesis of synaptic proteins, leading to impaired synapse function and facilitating neurodegeneration in a mouse model of FTLT (P301L tau) (Radford et al., 2015). Disrupted tau phosphorylation upon UPR activation was also found in these P301L tau transgenic mice (Radford et al., 2015). However, the mechanisms underlying this disruption are unclear. Interestingly, in contrast to several lines of tau transgenic mice, CHO-Tau35 cells did not exhibit obvious deposition of insoluble tau (Chapter 3). Therefore, the findings in this thesis provide new insight into how activation of the UPR might contribute to tau pathology in a cell model of tauopathies that is independent of tau mutations and aggressive tau aggregation. These results show that abnormal truncation of tau not only abolishes its physiological function, but also exert toxicity via the UPR pathway in the absence of apparent tau aggregation.

In mice expressing low amounts of Tau35, loss of synaptic protein and autophagic/lysosomal dysfunction were observed (Bondulich et al., 2016). The findings shown herein using the CHO-Tau35 cell model, provide additional clues to the potential molecular mechanisms underlying some of the phenotypes observed in Tau35 mice, as well as adding new information relevant to the pathogenesis of tau-

mediated neurodegeneration. A model describing these potential mechanisms is depicted in Figure 6.1.

First, truncation of the N-terminal half of tau may have a profound impact on the organisation of microtubules. In neurons, such abnormalities would significantly impair microtubule-based axonal transport (Chevalier-Larsen and Holzbaur, 2006). Compromised microtubule organisation undermines the integrity of the “rails” on which the molecular motors travel (De Vos et al., 2008; Millecamps and Julien, 2013). Reduced tubulin acetylation also decreases the interaction between kinesin-1 and microtubules (Perdiz et al., 2011; Reed et al., 2006), disrupting motor-protein trafficking, thereby affecting the transport of cargoes and organelles. Collectively, these pathways might synergistically lead to compromised delivery of molecular components that are critical for the maintenance and function of synapses and axons (De Vos et al., 2008; Neumann and Hilliard, 2014).

Figure 6.1 Tau35-induced neurodegeneration



The diagram shows potential mechanisms by which Tau35 could be involved in the development of neurodegenerative disease. Tau35 has a reduced ability to bind to and stabilise microtubules, leading to impaired axonal transport. Tau35 triggers UPR activation, which could inhibit Akt, resulting in GSK3β activation. Inhibited Akt is less responsive to insulin, and activated GSK3β in turn, can increase tau phosphorylation. GSK3β also contributes to axonal transport impairment, either directly through causing premature release of cargoes from molecular motors, or indirectly by activating HDAC6, which further reduces tubulin acetylation. UPR activation may also lead to cell death and autophagic dysfunction, which could in turn produce more Tau35, thereby generating a vicious cycle. Notably, Tau35 may also lack the ability of tau to modulate HDAC6 activity. These deleterious effects on proteostasis together give rise to degeneration of neurons. On the other hand, altered tau-membrane interaction may also contribute to the loss-of-function of Tau35. Events supported by the findings in this thesis are indicated in blue boxes. Events that need further verification are indicated in yellow boxes.

Activation of GSK3 β can also interfere with the molecular mechanisms underlying axonal transport, through premature release of transported cargoes (Millecamps and Julien, 2013; Stokin and Goldstein, 2006). The results described herein show that, although Tau35 retains an intact microtubule binding domain, without the presence of the N-terminal half of tau, it is unable to perform its physiological functions, in consistent with previous findings *in vitro* and in N2a cells expressing Tau-CTF24 fragment (Tau₂₄₃₋₄₄₁) (Jeganathan et al., 2006; Matsumoto et al., 2015). However, additional experiments are required to verify whether such deficits can recapitulate the pathological features of neurons expressing Tau35. Another intriguing question is how expression of Tau35 leads to decreased tubulin acetylation. One possible explanation is that activation of GSK3 β leads to increased HDAC6 activity, which deacetylates microtubules (Chen et al., 2010). Interestingly, there are reports suggesting that tau can inhibit HDAC6 through direct binding (Perez et al., 2009). Hence, another possibility is that Tau35 is unable to inhibit HDAC6 due to the lack of the N-terminal half of tau.

Secondly, the findings in this thesis raise the possibility that dysregulated insulin signalling may be a contributor to the development and pathogenesis of tauopathy, through increased tau phosphorylation. There is substantial experimental evidence that insulin resistance is capable of increasing A β and phosphorylated tau to initiate or exacerbate the pathological cascade associated with AD (Deng et al., 2009; Schubert et al., 2004; Stanley et al., 2016). It is known that impaired glucose metabolism is a characteristic feature in the pathology of AD (Schubert, 2005). Moreover, A β is a direct competitive inhibitor of insulin binding, thereby undermining the insulin signalling transduction (Matsumoto et al., 2015). Since Tau35 triggers dysregulation of Akt and GSK3 β , both of which are important components of the insulin signalling transduction cascade, this suggests that insulin signalling may also be disrupted by Tau35. Given the complex nature of the insulin signalling cascade, possible targets could include the insulin receptor, IRS1, and/or PI3 kinase, all of which lie upstream of Akt (Pessin and Saltiel, 2000).

Thirdly, the findings included here contribute to characterisation of the missing link between Tau35 and autophagic dysfunction, which could be related to activation of the UPR. Under ER stress, the UPR can either stimulate or inhibit autophagy (Cai et al., 2016). In most cases, the UPR stimulates autophagy to remove misfolded protein and restore cellular homeostasis (Rashid et al., 2015). However, in some pathological conditions including neurodegenerative disease, activation of the UPR

is aberrant and this can result in impaired autophagy (Rashid et al., 2015; Vidal et al., 2012). Indeed, a growing body of evidence has begun to shed light on the mechanisms that underlie ER stress-mediated inhibition of autophagy. For example, in a cell model of HD, as well as in a familial ALS-related mouse model, knock down of the IRE1-XBP1 axis elevates autophagy (Hetz et al., 2009; Hyrskyluoto et al., 2014; Vidal et al., 2012). XBP1 deficiency led to upregulated expression of Forkhead box O1 (FoxO1), a key transcription factor regulating autophagy in neurons (Vidal et al., 2012). These data indicate a potentially negative correlation between the activation state of the UPR and autophagy. Further evidence has shown that UPR activation perturbs normal autophagic flux via different mechanisms. Thus, IRE1 activation diminishes autophagic flux in HD via the IRE1-TRAF2 pathway (Lee et al., 2012). ER stress also upregulates the expression of secretory carrier membrane protein 5 (SCAMP5), inhibiting endocytosis, which in turn downregulates autophagic flux (Noh et al., 2009). Additionally, studies performed on muscle biopsies from patients with sporadic inclusion-body myositis revealed that UPR induction significantly decreased the activities of cathepsins D and B, increased LC-3II, and decreased expression of vacuolar H⁺-ATPase assembly protein 21 (VMA21) a chaperone for assembly of lysosomal V-ATPase (Nogalska et al., 2010). Depletion of VMA21 compromises V-ATPase activity and disturbs lysosomal pH (Ramachandran et al., 2009). These findings are of particular interest because similar changes in some of these proteins were noted in Tau35 mice (Bondulich et al., 2016).

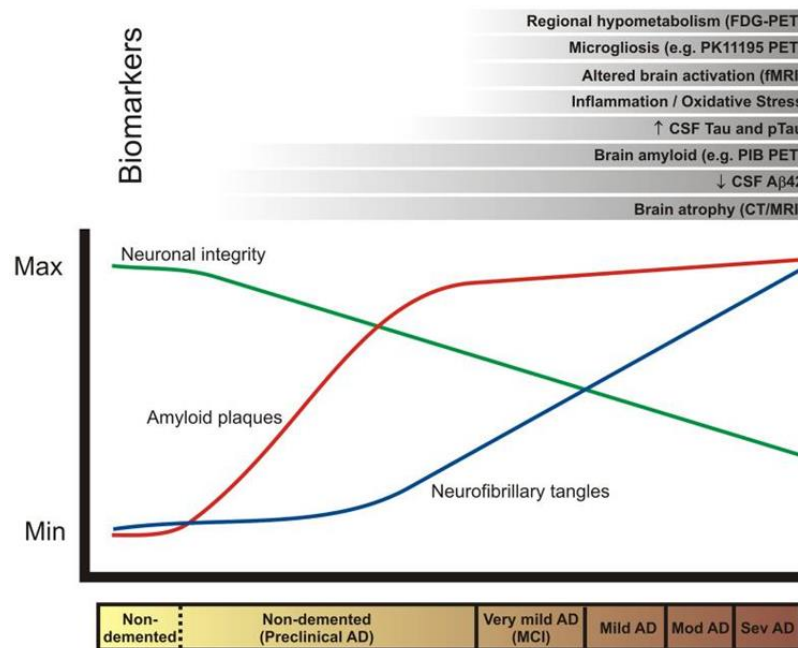
However, knowledge gaps remain concerning a comprehensive understanding of the mechanisms leading to autophagy impairment in the context of tauopathies. Given that several lysosomal proteases are capable of mediating tau truncation, the cause-effect relationship between autophagy-lysosome dysfunction and expression of Tau35 is another interesting question that has yet to be addressed. Notably, four of all the seven ubiquitination sites identified so far in full-length tau (Lee et al., 2013), namely Lys6, Lys11, and Lys48 Lys63, Lys254, Lys311, and Lys353, are absent from Tau35. Therefore, it is unclear whether Tau35 undergoes the same pattern of degradation as full-length tau. An altered pattern of degradation for Tau35 could also contribute to autophagy-lysosome dysfunction in Tau35 mice and in CHO-Tau35 cells.

Finally, the occurrence of UPR activation in CHO-Tau35 cells, may partially explain the apparent temporal mismatch between the onset of neurodegeneration and the

accumulation of NFTs in human tauopathy (Fig. 6.2) (Gómez-Isla et al., 1997; Kimura et al., 2010; Spire-Jones et al., 2009). Importantly, Tau35 is able to exert detrimental effects to cells without any evidence of the formation of insoluble deposits of tau, indicating that soluble tau species, and possibly also small tau oligomers, may be the actual toxic form(s) of tau in these conditions. These findings are consistent with data obtained from other models of tauopathy, such as *Drosophila* and in neurons expressing P301L tau (Cowan and Mudher, 2013; Kopeikina et al., 2012). The UPR is also demonstrated to be a significant player in FTLD-tau related neurodegeneration, particularly in relation to synaptic impairment and cognitive defects. Since aberrant activation of the UPR will result in the perturbation of other physiological processes within cells, in turn driving further pathological changes, it would appear that even a minimal amount of pathological tau is sufficient to trigger the disease cascade. These results are therefore in line with previous findings using transgenic mouse models exhibiting low expression of disease-related mutant tau species (Dawson et al., 2007; Maeda et al., 2016; Rosenmann et al., 2008). However, whilst a number of unanswered questions remain, the results herein shed light on potential mechanisms that could underlie the neurodegeneration in sporadic tauopathies in which no aggressive tau mutants are expressed (Morris et al., 2002; Reeves, 2005; Yancopoulou and Spillantini, 2003). Importantly, sustained intrinsically activation of PERK in CHO-Tau35 may also be of help to explain the loss of synaptic protein found in Tau35 transgenic mice, as it is proven that over-activation of the PERK branch of the UPR directly contributes to disease pathogenesis through the reduction in the synthesis rates of critical neuronal protein in AD related mouse model (*APP* K670N/M671L, *PSEN1* ΔE9) as well as FTLD-tau related mouse model (P301L tau) (Ma et al., 2013; Radford et al., 2015).

Taken together, this thesis demonstrates that N-terminal truncation of tau promotes tau pathology via both loss-of-function and gain-of-toxicity routes. Moreover, GSK3β and UPR activation may be important links that bridge the presence of Tau35, and possibly other similarly truncated tau species, with downstream cell abnormalities and pathology.

Figure 6.2 Temporal relationship between molecular biomarkers and neuropathology and clinical changes in Alzheimer's disease



The graph shows the temporal relationship between the development of clinical and neuropathological features of AD, and the emergence of biomarkers. FDG-PET: ^{18}F -Fluorodeoxyglucose positron emission tomography; PET: Positron emission tomography; fMRI: Functional magnetic resonance imaging; CSF: Cerebrospinal fluid; CT: Computed tomography; MRI: Magnetic resonance imaging; (Craig-Schapiro et al., 2009).

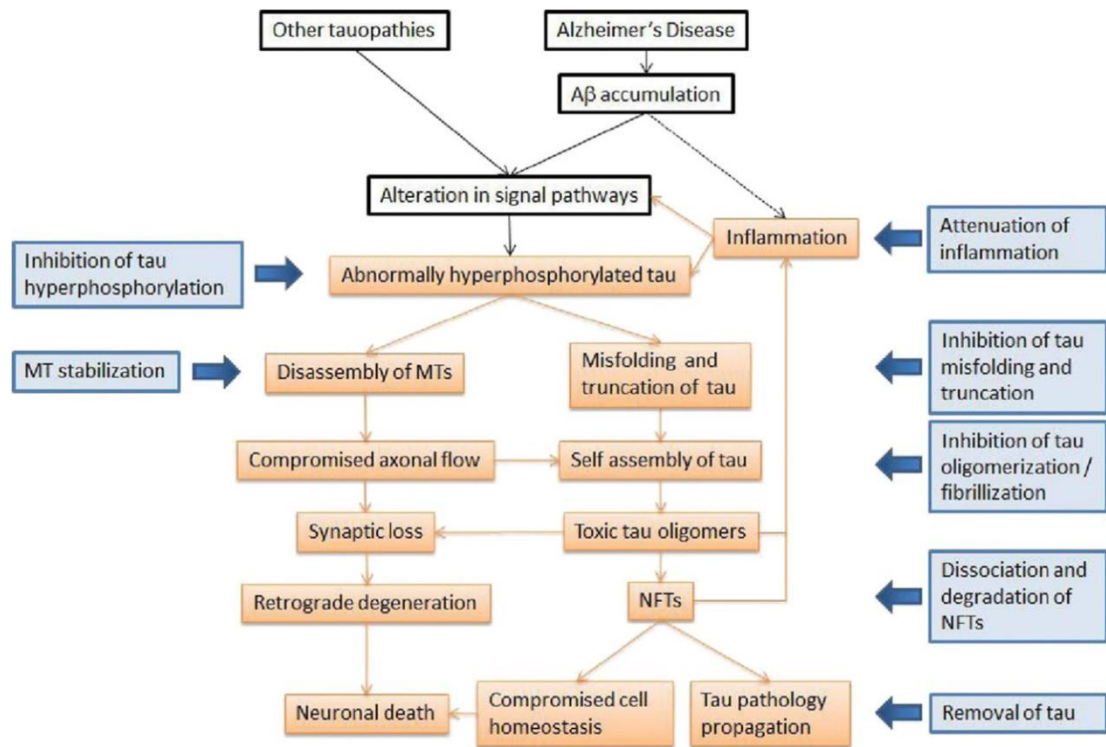
6.2 Therapeutic perspectives

Significant effort has been devoted to the identification of therapeutic treatments that might result in the cure of AD and other tauopathies. The primary therapeutic approaches that have been considered include increasing microtubule stabilisation, inhibiting tau phosphorylation, enhancing clearance of misfolded tau or NFTs, inhibiting propagation of tau pathology, tau immunisation, and attenuating inflammatory pathways, as summarised in Figure 6.3 (Yoshiyama et al., 2013). Although the evidence for tau-based treatments for AD is encouraging, additional work is undoubtedly needed to optimise each treatment strategy for the successful development of safe and effective therapeutics.

6.2.1 Effects of GSK3 inhibition on tau pathology

As mentioned in Chapter 1, elevated tau phosphorylation is regarded as a prerequisite for the progression of tau pathology, most likely mediated by one or more protein kinases, that could include GSK3, Cdk5, CK1, PKA, CaMKII, MAPKs (e.g. ERK1/2, JNKs and p38), and MARKs (Martin et al., 2011). Indeed, kinase inhibitors are being actively pursued in the pharmaceutical industry for numerous clinical applications. In addition to its function as a tau kinase, GSK3, especially GSK3 β , is a pivotal player in the pathogenesis of tauopathies through various mechanisms. However, although inhibiting GSK3 is theoretically promising for the development of therapeutic interventions, no convincing positive results have yet been acquired from clinical trials of GSK3 inhibitors. Hence, new strategies may be needed to fully exploit the potential of GSK3 and potentially other tau kinases as a drug target.

Figure 6.3 Possible therapeutic targets in tauopathies



The diagram shows the potential relationships between Aβ: Amyloid-β protein; MT: microtubules; NFTs: neurofibrillary tangles; Tauopathies: a class of neurodegenerative diseases associated with the pathological aggregation of tau protein in neurofibrillary or gliofibrillary tangles (Yoshiyama et al., 2013).

Research into the use of lithium as GSK3 inhibitor has been carried out for many years. Lithium ions inhibit both GSK3α and GSK3β directly through competitive inhibition of Mg^{2+} , with an inhibitory constant (Ki) of 2 mM (Klein and Melton, 1996; Ryves and Harwood, 2001). The potency of lithium as a GSK3 inhibitor has been proven in both cell and mouse models of tauopathies. In Ntera2/D1 neuron-like cells, lithium reduces tau phosphorylation (Hong et al., 1997; Lovestone et al., 1999; Munoz-Montano et al., 1997; Takahashi et al., 1999) and APP processing (Phiel et al., 2003; Su et al., 2004; Sun et al., 2002). Inhibition of GSK3 by lithium also protects cortical and hippocampal primary neurons against Aβ-induced neurodegeneration (Alvarez et al., 1999), suggesting that lithium may have important implications for AD. However, this notion has been challenged by the finding that treatment with lithium increases β-secretase activity, and subsequently

increases extracellular A β in CHO cells and in rat cortical neurons (Feyt et al., 2005). Nevertheless, in this study, treatment with SB415286, another GSK3 inhibitor, resulted in a slight decrease in A β production, suggesting that increase A β production is GSK3-independent (Feyt et al., 2005). Administration of LiCl for one month to transgenic mice overexpressing mutant human tau (P301L, 0N4R isoform), reduced both tau phosphorylation and the amount of insoluble tau (Noble et al., 2005; Pérez et al., 2003). More recently, by using a double transgenic mouse overexpressing both GSK3 β and tau carrying a triple FTLD-tau mutation (G272V, P301L and R406W), it was demonstrated that LiCl administered to pre-symptomatic animals for 7.5 months, prevented the increase in tau phosphorylation and the onset of tau pathology (Engel et al., 2006). LiCl administration inhibited of tau phosphorylation in mice with existing tau pathology, but NFTs persisted. Effects comparable to LiCl treatment were also found in mice with downregulated GSK3 β expression, suggesting that the effects of LiCl were specifically due to inhibition of GSK3 β (Engel et al., 2006). Finally, in 3 \times Tg-AD mice (triple transgenic mice expressing three proteins with mutations associated with familial AD: *APP* K670N/M671L, *MAPT* P301L, and *PSEN1* M146V), which display both A β plaques and tau fibrils in their brains, LiCl treatment of 15 month-old transgenic mice for 1 month led to reduced tau phosphorylation, without affecting the A β plaque burden (Caccamo et al., 2007). However, LiCl was unable rescue the memory deficits in these animals. Lithium has also been shown to prevent A β toxicity (Sofola et al., 2010), preserve dendritic structure (Rockenstein et al., 2007), facilitate neurogenesis (Fiorentini et al., 2010), and rescue A β -induced cognitive impairment (Fiorentini et al., 2010; Rockenstein et al., 2007; Toledo and Inestrosa, 2010). These findings have proposed lithium as a likely candidate for future therapeutic applications in the treatment of tauopathies.

However, despite the *in vitro* and *in vivo* studies revealing a beneficial effect of lithium on tau and A β pathology, observational studies and other reports yield conflicting outcomes. Amongst the sparse studies of LiCl in patients, some data suggest lithium might have disease-modifying properties in AD, and perhaps other tauopathies, most likely through GSK3 inhibition (Forlenza et al., 2011; Havens and Cole, 1982; Terao et al., 2006), whereas others report either no or limited effects of LiCl treatment (Hampel et al., 2009; Macdonald et al., 2008). This discrepancy is probably due to the nature of lithium as a non-specific GSK3 inhibitor. Lithium also inhibits other targets, including inositol monophosphatase (Berridge et al., 1989), other phosphomonoesterases structurally related to inositol monophosphatase

(Phiel and Klein, 2001; York et al., 1995), phosphoglucomutase (Ray et al., 1978), and possibly other enzymes.

In recent years, a number of potent and relatively selective, small molecule GSK3 inhibitors, as well as phosphopeptides that act as GSK3 inhibitors, from different chemical families, have been described (Table 6.1). Some of these GSK3 inhibitors are currently being tested in phase I proof-of-concept clinical trials, while others are in phase II clinical trials for the treatment of both AD and PSP (Medina et al., 2011).

Table 6.1 Selected GSK3 inhibitors in clinical development for the treatment of tauopathies

Drug	Institution	Development phase	Indication
Tideglusib (NP-12)	Noscira	Phase II	AD, PSP
Lithium	University of Sao Paulo, Brazil	Phase II	AD
Valproic acid	Nantes University Hospital, France	Phase II	PSP
AZD-1080	AstraZeneca	Discontinued	AD

AD: Alzheimer's disease; PSP: Progressive supranuclear palsy; Phase II: Second phase of clinical research (testing of candidate drug on patients to assess efficacy and safety) (Medina et al., 2011).

6.2.2 Inhibiting GSK3 to restore axonal transport

The results shown in Chapter 4 suggested a potential link between GSK3 and the cellular machinery governing microtubule dynamics. Similarly, inhibition of GSK3 β by LiCl promotes axon outgrowth and stimulates the formation of polarity in mammalian neurons (Jiang et al., 2005; Munoz-Montano et al., 1999; Yoshimura et al., 2005). This notion is further substantiated by the finding that HDAC6, a tubulin deacetylase, is regulated by the Akt-GSK3 pathway (Fig. 6.1) (Chen et al., 2010). In hippocampal neurons, inhibition of HDAC6 increases acetylation of tubulin and the association of kinesin-1 with mitochondria. Comparable effects are obtained by inhibiting GSK3 β . Furthermore, inhibition of GSK3 β decreases the amount and activity of HDAC6, whereas when GSK3 β activity was elevated by inhibiting Akt, the

activity of HDAC6 was significantly increased. Importantly, GSK3 β co-localises with HDAC6 and phosphorylates HDAC6 at Ser22 (Chen et al., 2010). It is worth noting a recent report of LiCl activating MEC-17, a tubulin acetyl transferase, by inhibiting GSK3 β , and promoting α -tubulin acetylation, which resulted in elongation of the primary cilia in human fibroblast KD cells (Nakakura et al., 2015). Tubulin acetylation is an important modification that mediates recruitment of molecular motors to microtubules, which is a key event during microtubule based cargo delivery. These findings suggest the potential of GSK3 inhibition in restoring deficits in axonal transport, possibly through modulating tubulin acetylation.

6.2.3 Targeting the UPR

Correlations between UPR activity and neurodegenerative disease are accumulating in recent years and hence, modulating components of the UPR cascade is receiving increased attention for the potential treatment of neurodegenerative disease (Lindholm et al., 2006; Ma and Klann, 2014; Salminen et al., 2009; Scheper and Hoozemans, 2013; Stefani et al., 2012).

Of the three UPR branches, the PERK and IRE1 branches have been extensively described as being involved in several models of neurodegeneration such as AD, PD, HD, ALS and CJD (Ferreiro and Pereira, 2012). On the other hand, given that upstream signals of the UPR may be amplified during the transduction process, a subtle inhibition of factors involved in the initiation stage of UPR is believed to be able to induce significant therapeutic effects. Hence, inhibiting PERK and IRE1 has been proposed as a potential therapeutic strategy for neurodegenerative disease (Maly and Papa, 2014).

PERK inhibition, either through direct knockout of the PERK gene or using small molecule inhibitors, is able to alleviate the progression of neurodegeneration (Ma and Klann, 2014). Deletion of the PERK gene restores the defect in the translation of synaptic proteins and rescues the neurodegenerative phenotype, including deficits in plasticity, neuronal cell death, and memory deficits (Ma et al., 2013; Moreno et al., 2012). On the other hand, treatment with GSK2606414, an ATP-competitive small molecule inhibitor of PERK, ameliorated neurodegeneration, similar to genetic interference with the PERK pathway (Moreno et al., 2013). These findings provide a promising proof-of-concept for the effectiveness of PERK inhibition as a feasible therapeutic intervention in disorders in which the PERK branch of the UPR is activated. However, both inhibition strategies of PERK are associated with severe

side effects. Homozygous PERK^{-/-} mice displayed phenotypes including severe disruption in the development and physiology of the animals, very similar to humans with Wolcott–Rallison syndrome, caused by mutations in the gene encoding PERK (*EIF2AK3*) (Delepine et al., 2000). A pronounced defect in the function of the endocrine and exocrine pancreas was also observed, resulting in many systemic problems and early mortality (Harding et al., 2001; Zhang et al., 2002). PERK-deficient cells are also more sensitive to ER stress (Harding et al., 2000). Similarly, severe pancreas pathology was also observed in the animals treated with GSK2606414 (Moreno et al., 2013).

Integrated stress response Inhibitor B (ISRIB) has been identified as a small molecule which inhibits signalling downstream of eIF2 α , and is proven to enhance spatial and fear-associated learning in wild-type mice, without reported toxic side effects *in vivo* (Sidrauski et al., 2013; Sidrauski et al., 2015). More importantly, in mice with prion-disease, ISRIB treatment confers neuroprotection without pancreatic toxicity. In contrast to GSK2606414, which completely restored protein synthesis, ISRIB was able to rescue only ~70% of protein synthesis in prion-diseased mice (Halliday et al., 2015). However, interestingly, this partial restoration of protein synthesis was sufficient to protect neurons and to increase the life-span of ISRIB-treated prion-diseased mice, with strongly reduced pancreatic toxicity, compared to treatment with GSK2606414 (Halliday et al., 2015).

Based on the progress and setbacks during PERK inhibition, targeting eIF2 α could represent a more promising therapeutic approach for neurodegeneration. As the downstream target of PERK, the pathway through which eIF2 α -mediated translational repression overlaps with PERK-induced attenuation of translation (Scheper and Hoozemans, 2015). Mechanistically, eIF2 α is the actual executor of PERK-mediated chronic inhibition of translation. Over-activated eIF2 α is closely associated with several neurodegenerative diseases including prion diseases, tauopathies and sporadic ALS (Ma et al., 2013; Moreno et al., 2013; Moreno et al., 2012; Segev et al., 2013). It has been proposed that aberrant activation of eIF2 α compromises *de novo* protein synthesis for long-term synaptic plasticity and memory formation, leading to memory loss (Chen et al., 2003). Correspondingly, in transgenic mice, reducing phosphorylation of eIF2 α by heterozygous expression of an eIF2 α phospho-null mutant lowers the threshold for long-term potentiation, enhancing synaptic plasticity, spatial learning and memory in these animals (Costa-Mattioli et al., 2007). Furthermore, the rescue effect of PERK knockout in APP/PS1

mice (expressing APP K670N/M671L and PSEN1 L166P), appears to be more closely related to eIF2 α than to PERK, because deletion of GCN2 (another eIF2 α kinase) has the same effect (Ma et al., 2013). These findings further emphasise repression of eIF2 α -mediated protein translation as a key target for UPR-based treatment interventions.

What should not be overlooked is that the UPR is a highly regulated process involving crosstalk between its three branches. Thus, UPR components other than PERK and eIF2 α , could also hold potential as drug targets. In a superoxide dismutase 1 (SOD1) mouse model of familial ALS, XBP1 deficiency is protective due to enhanced autophagic clearance of SOD1 aggregates (Hetz et al., 2009). Similarly, in a transgenic model of HD, deletion of XBP1 was also found to be protective and this was accompanied by increased autophagic clearance of the huntingtin aggregates (Vidal et al., 2012). Further to this, inhibitors of IRE1 could also be a prospective intervention target. Given the dual function of IRE1 as an RNase and a kinase, three types of inhibitors have been developed. Type I kinase inhibitors that inhibit autophosphorylation of IRE1, but has no effect on RNase activity, have not yet shown any utility for clinical development (Wang et al., 2012). Type II inhibitors inhibit both kinase and RNase activities and thus effectively block all signalling through IRE1 (Ghosh et al., 2014). In models of ER stress-mediated degeneration, the type II IRE1 inhibitor, KIRA6 promotes cell survival (Ghosh et al., 2014). Type III inhibitors inhibit only the RNase activity of IRE1 (Cross et al., 2012; Sanches et al., 2014), and these could exert protective effects through blocking the generation of activated XBP1.

Taken together, this work highlights some of the possible intervention strategies for the treatment of tauopathies, which are closely relevant to the findings reported in this thesis. Due to its versatility in mediating tau pathology, inhibiting GSK3 has not only emerged as a promising therapeutic approach, but also served as a valuable tool for understanding the pathogenesis of these diseases. The UPR, on the other hand, whose role in driving neurodegeneration has only recently been elaborated, has provided multiple targets for drug discovery (Scheper and Hoozemans, 2015). Nevertheless, despite some significant progress being made, knowledge gaps still remain in the understanding of the mechanisms that cause neurodegeneration. Firstly, given that different subtypes of tauopathies possess similar, yet distinct, pathological features, it is reasonable to speculate that there might be different mechanisms that correlate with each disease subtype. However, most GSK3

inhibitors are tested in models either with accumulated A β or expressing aggressive tau mutations (Lei et al., 2011; Medina et al., 2011). Thus, the effectiveness of such GSK3 inhibitors in models of sporadic tauopathies still needs validation. Secondly, although co-localisation of GSK3 and HDAC6 has been observed, direct evidence showing a direct interaction between these two proteins has not yet been shown. Additionally, the precise molecular details of the interplay between deacetylation by HDAC6, acetylation by MEC-17 and phosphorylation mediated by GSK3, are unknown. Thirdly, most current UPR-targeting strategies aim to restore protein synthesis by modulating the PERK branch of the UPR (Smith and Mallucci, 2016). However, repression of protein synthesis is not the only detrimental effects that resulting from aberrant activation of the UPR, nor is the PERK branch the only signalling cascade that activates the UPR. Thus, the potential of strategies that target other aspects of the UPR, such as cell death and insulin resistance, as well as modulators targeting other branches of the UPR, such as IRE1 and ATF6, are not yet fully explored. Therefore, the CHO-Tau35 cell model of tauopathies, described herein, together with the Tau35 transgenic mouse model can serve as useful experimental platforms in which to address these outstanding questions.

6.3 Concluding remarks

In conclusion, in this thesis, using a cell model stably expressing Tau35, a disease-associated and N-terminally truncated, tau fragment, multiple pathological changes can be detected due to expression of Tau35. These results add new information to current paradigms aiming to explain the mechanisms underlying the pathogenesis of tau-mediated neurodegeneration. The work described here provides evidence that this new CHO-Tau35 cell model can be used to explore the role of tau truncation in tauopathy and could provide a useful tool for the assessment and potential development of novel therapeutic interventions.

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